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FINAL REPORT

19970258

DEVELOPING HULLESS BARLEY WITH RESISTENCE TO COVERED SMUT AND FALSE LOOSE SMUT THROUGH BIOTECHNOLOGY

Funded by
The Agriculture Development Fund

January 2003
Prepared by: University of Saskatchewan

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103-02846/

Developing Hulless Barley with Resistance to Covered Smut and False Loose Smut through Biotechnology

Terminal Report on ADF Project # 97000258

December 31st 2002

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(a) Abstract

The objectives were to develop molecular methods that would facilitate screening for two seed-borne smuts and to use these methods to develop hulless barley with resistance. Early experiments failed to differentiate between the two forms of smut. Inoculation techniques for covered smut were developed and hulled lines with good levels of resistance were identified. Four molecular markers linked to the resistance of line Q21861 were identified. RAPD marker $OPJ10^{450}$ was converted into a SCAR marker and mapped to the short arm of chromosome 5(1H). These markers were validated on hulless x hulled populations. The SCAR marker is being used in marker-assisted selection for covered smut resistant hulless barley.

(b) Executive Summary

Covered smut/false loose smut of barley is caused by *Ustilago hordei/U. nigra* and found everywhere barley is grown. The diseases cause yield reductions in Canada, ranging from 0.2 % in 1991 to 0.8 % in 1989 and contaminate healthy seeds with black teliospores. They can be effectively controlled by fungicidal seed treatment, sowing disease free seeds and growing resistant cultivars. Seed treatment with fungicides is effective but the fungi may develop resistance to the fungicides. Induced mutants tolerant to four different fungicides have been reported. Fungicides also require producers to incur an additional cost and they may have an adverse effect on the environment, on other organisms and the operator. Therefore, growing of resistant cultivars would be the most effective, economical and eco-friendly method to control these diseases.

Breeding for resistance to seed-borne smuts is not routine because screening is time consuming. The time required between inoculations and symptom development is 2-3 months. Screening is also space and labour consuming thus limiting the size of a breeding program. In addition, inoculation is inconsistent as even in inoculated highly susceptible lines some disease-free plants may occur.

Hulless barley is becoming a significant crop in western Canada, as it is an appropriate feed grain for the expanding hog industry. Hulless barley cultivars with acceptable yield and quality have been developed but resistance to diseases such as seed-borne smuts needs to be incorporated. Seed treatment with systemic fungicides adversely affects germination of hulless barley and hulless barley is more pre-disposed to infection than hulled types. As large amounts of hulless barley become used for food & feed and with the demand for organic product, disease resistance is essential as the use of chemical seed treatment is precluded. Techniques that aid in selection for resistance, such as molecular markers, would be of great value to hulless barley breeding programs. Molecular markers facilitate breeding for disease resistance as they can be used to screen at any stage of growth and are not influenced by the environment. In addition, resistance can be selected for in the absence of the pathogen.

The main objectives of this project were:

1. Standardization of artificial inoculation techniques
2. Identification of sources of resistance to covered smut and development of segregating populations including hulless parents
3. Development of molecular markers in segregating populations with RAPD or AFLP technology using Bulked Segregant Analysis (BSA)
4. Use of these markers for Marker Assisted Selection (MAS) of smut resistant hulless barley

Based on early studies of colony morphology, DNA sequence of the ITS regions, disease symptoms and electron micrographs, *U. hordei* (covered smut) and *U. nigra* (false loose smut) appeared to be synonymous species, therefore, the research focused on only covered smut.

1. Standardization of artificial inoculation techniques

The artificial inoculation technique was standardized with some modifications of the spore-suspension technique of Tapke and Bever (1942). Seeds were shaken vigorously for 30 seconds in a spore suspension consisting of 1 g of *U. hordei* teliospores in 1 L of double distilled water. Following shaking, the seeds were incubated at room temperature for 15 minutes; the suspension was then decanted off. Two disease evaluation methods were used to evaluate field material (visual rating and percent-infected heads) and were highly correlated. Visual rating was more efficient while percent-infected heads gave more detailed information. Poor emergence and emergence of short, thick and distorted seedlings was sometimes observed following inoculation of hulless barley.

1) It was found that the inoculated seeds could be planted immediately or left to dry and planted at convenience.
2) Conditions under which the inoculated seed was stored, including humidity and temperature had no significant effect on the degree of smut infection and inoculated seeds could be stored for 6-12 months without affecting the degree of smut infection.
3) Three year-old inoculum gave the same level of infection as fresh inoculum.
4) Date of seeding in the field significantly affected covered smut infection. There was more disease on plants sown on 23rd May and 6th June than those sown on 9th May, therefore early seeded crops may develop less covered smut.
5) Dehulling of the barley seeds from resistant cultivars did not significantly reduce their resistance to covered smut suggesting that the hulless condition does not play a major role in reduced resistance although there was some evidence it may play a minor role.
6) The reaction of a set of differentials for covered smut of barley (lines with known reactions to certain races) indicated that the inoculum being used was a mixture of 2 or 3 races.

2. Identification of sources of resistance to covered smut, development of segregating populations and inheritance studies

Many hulled and hulless genotypes from the CDC, Saskatoon and AAFC, Brandon barley breeding programs were screened for their resistance/susceptibility to covered smut.

1) Many hulled cultivars/lines (Q21861, CDC Dolly, TR244, TR640, TR255, CDC Helgason, CDC Bold and TR246) showed good levels of resistance to covered smut in both the field and greenhouse.
2) No cultivar/line was completely resistant.

Segregating populations were developed by crossing these resistant cultivars with susceptible/moderately susceptible hulless/hulled cultivars and using doubled-haploid, single seed decent (SSD) or regular pedigree breeding methods. Inheritance of resistance in these cultivars was studied by inoculating and then planting the segregating populations in the field for either 2 or 3 years. The reaction of putative resistant lines was confirmed in greenhouse trials (to ensure they were not escapes).

1) A single major gene for resistance was found to condition resistance in Q21861 based on three years screening of the doubled-haploid population Q21861/SM89010 (Q/SM) in the field and two seasons of screening in the greenhouse.
2) Segregation of resistant and susceptible lines in the doubled-haploid population HB328/TR244 also indicated a single major gene for resistance in TR244 to the mixture of isolates used in this study. It was confirmed in other two populations viz. TR337/TR244 and CDC Gainer/TR244.
3) A single gene for resistance was found in TR246 using the cross SB93763/TR246.
4) In the cross CDC Dolly/ RFLP Harrington, the resistant and susceptible lines segregated in a 1:3 ratio indicating two recessive genes for resistance in CDC Dolly.
5) Segregation of HB334/Manley population revealed that the resistance gene (s) present in HB334 and Manley were different. Some lines with higher level of resistance than either of the parents indicates additive gene effects.
6) Segregation of three hulless x hulled populations i.e. CDC McGwire/TR640, CDC McGwire/Q21861 and CDC Candle/Q21861 in a 1: 1 ratio indicated a single major for resistance in TR640 and Q21861.

3. Identification and development of molecular markers

The Q/SM population was used to develop molecular markers with RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragment Length Polymorphism) primers using BSA. Of the 440 10-mer random primers tested, primer OPJ10 resulted in a reproducible bright polymorphic band of ~450 bp present in the susceptible bulk, the susceptible parent (SM89010) and each individual of the susceptible bulk but not in the resistant bulk, resistant parent (Q21861) and resistant individuals. The entire population was screened with this primer. The marker $OPJ10^{450}$ was tightly linked (2.4 cM) in repulsion to covered smut resistance of Q21861.

All of the AFLP primer pairs screened (82) produced scoreable banding patterns. However, only one primer pair E-AAA/M-CCC produced a polymorphic band of 265 bp in the resistant bulk, resistant parent and all individuals constituting the resistant bulk.

This band was not present in the susceptible bulk, susceptible parent or any of the susceptible individuals. The entire population was screened with this primer pair. Eleven of 127 individuals were recombinants between this marker and the resistance gene suggesting a linkage of this marker in coupling.

The close linkage of the RAPD marker ($OPJ10^{450}$) was good for marker-assisted selection for covered smut resistance in a breeding program. However, the RAPD technique is sensitive to reaction conditions, which results in poor reproducibility. To overcome this problem, longer primers can be developed from RAPD fragments (Paran and Michelmore 1993) and these longer primers generate a sequence characterized amplified region (SCAR). SCARs are advantageous over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions and they can be potentially converted into allele specific markers. The polymorphic band produced by the primer OPJ10 in the susceptible genotype was excised, cloned and sequenced. Two primers were developed from the amplified sequence. The primer pair ULR1-F and ULR-R yielded a major band of 450 bp that was present in the resistant genotypes and absent in the susceptible genotypes. The polymorphic banding pattern was reproducible and the entire population was screened with these SCAR primers.

All genotypes negative (no band) for the $OPJ10^{450}$ RAPD marker were positive (a major band of 450 bp) for the SCAR marker and all genotypes positive for the $OPJ10^{450}$ RAPD marker were negative for the SCAR marker. The SCAR marker developed in the Q/SM population was also present in TR640 (also resistant to covered smut).

AFLP analysis of CDC Dolly/RFLP Harrington and TR244/HB328 populations was also carried out using BSA. Many primer-pairs gave polymorphic bands between the resistant and susceptible bulks and resistant and susceptible parents, however, the putative markers were not closely linked to covered smut resistance. Similarly, we could not find any closely linked marker by using SSR (Simple Sequence Repeats) and ISSR (Inter-simple Sequence Repeats) primers.

4. Mapping and utilization of molecular markers for MAS

The genetic map of barley is well developed hence the RAPD marker $OPJ10^{450}$ identified from the Q/SM population was mapped using the Harrington/TR306 mapping population as this would identify other markers in this region that might be useful as markers for the covered smut gene. $OPJ10^{450}$ mapped to the short arm of chromosome 5 (1H).

One PCR- based marker (aHor2) has been mapped to this region of chromosome 5. It produces a co-dominant polymorphism with bands at 800 bp and 1100 bp depending on genotype. This marker was used to screen the parents of covered smut segregating populations and it was polymorphic between many parents viz. Q21861 and SM89010, Q21861 and CDC Candle, CDC Dolly and RFLP Harrington, Manley and HB334.

1) aHor 2 was found to be closely linked to the covered smut resistance of Q21861 and it co-segregated with the SCAR marker UhR 450 with few exceptions.

2) A RAPD marker $OPO6^{780}$, present on short arm of chromosome 5, was also found to be closely linked to covered smut resistance in Q21861.
3) Three markers (UhR 450, aHor 2 and $OPO6^{780}$) were validated on three covered smut segregating populations viz. Q21861/CDC Candle, CDC McGwire/TR640 and CDC McGwire/Q21861.
4) The markers UhR 450 and $OPO6^{780}$ were linked to covered smut resistance in two populations (CDC Candle/Q21861, CDC McGwire/Q21861) and loosely linked in CDC McGwire/TR640 population. aHor 2 was polymorphic only in CDC Candle/Q21861 and linked to covered smut resistance in this population.
5) The SCAR marker, UhR 450 is robust and worked well with the rapid leaf-disc template preparation method.

These markers will be useful for marker-assisted selection for covered smut resistance in hulled and hulless barley breeding programs.

Additional Experiments:

1) Work is in progress to introgress both loose smut and covered smut resistance into CDC McGwire, a high yielding hulless line, through MAS backcrossing and doubled haploidy.
2) Resistant by resistant crosses has been made and populations were developed through SSD to identify whether the gene/genes present in resistant cultivars identified to date are different.
3) Many barley genotypes have been screened against different isolates of *U. hordei* with known virulence/avirulence genes. Many lines showed differential reactions to different isolates suggesting that they have different resistance genes and some lines have more than one resistance gene.
4) Screening of Harrington/TR306 DH population with a specific isolate (Uh2-V1V1v2v2v6v6) revealed a single gene (*UhR1*) in TR306.
5) In cross HB328/TR244, the resistant and susceptible lines segregated in a 7:1 ratio indicating three genes for resistance in TR244 to the isolate Uh6 (v1v1v2v2V6v6).

Technical Report

1. Standardization of artificial inoculation techniques

The artificial inoculation technique was standardized with some modifications of the spore-suspension technique of Tapke and Bever (1942). Seeds were shaken vigorously for 30 seconds in a spore suspension consisting of 1 g of *U. hordei* teliospores in 1 L of double distilled water. Following shaking, the seeds were incubated at room temperature for 15 minutes; the suspension was then decanted off. It was found that the seeds could either be planted immediately or planted after drying. The latter is necessary for field planting.

Some additional experiments were conducted to see the effect of seeding date, space planting, inoculum age, time of inoculations and dehulling on the development of covered smut.

1.1 Effect of Seeding Date

Objective: To investigate the effect of date of sowing on the level of infection.

Five cultivars were used for this experiment viz. CDC Dolly 6, HB328, Q21861, RFLP Harrington and CDC Candle. CDC Candle and HB328 are hulless whereas others are hulled. Six hundred seeds of each genotype were inoculated for each date of seeding with spore suspension technique using mixture of isolates of *U. hordei*. There were three dates of seeding at 2 weeks intervals i.e. May 9, 2000; May 23, 2000 and June 6, 2000 in the Preston Fields.

There were 12 replications for each genotype and they were completely randomized. In each replication, there are four hills and in each hill 10 seeds were sown. Infected heads and total number of heads were counted in each hill and per cent infected heads were calculated. Statistical analysis was done using SAS program.

The results clearly showed that covered smut incidence was significantly less in plants sown on 9th May than that of 23rd May and 6th June sown plants (Table 1) and the covered smut incidence in the last two dates were statistically at par. The effect of seeding date was more profound in hulless barley cultivars (CDC Candle and HB328) than that of hulled ones. The mean average temperature was 6.3°C for the week following first date of seeding whereas it was 12.3°C and 13.0°C for the weeks following second and third date of seeding, respectively. Secondly, the minimum temperature was below 0°C for many days after the first date of seeding which was not conducive for germination and growth of the pathogen. It has been reported earlier that germination temperature has the significant effects on the level of infection (Tapke, 1948). It may be concluded that to have good level of covered smut incidence, sowing should be done in second fortnight of May or first week of June or in other words when the mean temperature is more than 10°C. On the other hand side, the farmers are advised to sow their crop early to escape the covered smut incidence.

Table 1: Effect of date of seeding on covered smut infection

Date of seeding	Per cent infected heads					Mean
	CDC Candle	RFLP Harrington	HB328	CDC Dolly	Q21861	
09-May-00	28.8	11.0	9.4	0.1	0.0	9.9
23-May-00	36.8	13.4	22.1	0.6	0.3	14.6
06-Jun-00	30.1	14.2	24.0	0.5	0.8	13.9
Mean	31.9	12.8	18.5	0.4	0.4	

LSD for dates - 2.5
LSD for cultivars - 3.2
LSD for dates *cultivars - 5.6

Therefore, the covered smut nursery was always planted in the end of May or first week of June. The summers of 2001 and 2002 were dry and hot, particularly in June and July and drought was very severe in some parts of Saskatchewan and Alberta. The prevalence and severity of many diseases particularly foliar diseases was less even under artificial epiphytotic conditions. However, it did not affect the severity of covered smut, levels were as high as or higher than the previous years e.g.

	% infected heads in field			
	1999	2000	2001	2002
CDC Candle	44.4	32.9	48.2	49.9

The date of seeding significantly affects covered smut severity. Once the spores cause infection, the pathogen becomes systemic and grows with the plant and thereafter weather does not have much influence.

1.2 Space Planting Experiment

Objective: To record the disease reaction on a plant basis, as it is very difficult to record the data on a plant basis from a normal sowing in the field.

Four cultivars were selected for this exp. viz. CDC Candle, RFLP Harrington, Q 21861 and TR244. Sixty-gram of seed of each genotype were inoculated with *U. hordei* and planting was done with the space planter. There were two rows of each cultivar. Row length was 180 ft (approx.) and seed to seed distance was 4 to 6 inches. There was problem in planting this exp. as only 16 plants of TR244 were emerged. A plant showing one or more smutted heads was considered to be infected. The disease scoring is always

lower when measured as per cent infected heads than that of per cent infected plants because there are many smut free heads even in the infected plants.

Space planting did not affect the level of infection we were getting with normal planting, however, it needed more space. Therefore, space planting was not followed in the future experiments.

Table 2: Incidence of covered smut in space planting

Genotype	Infected plants	Total plants	% infected plants
Q21861	0	137	0.0
TR 244	0	16	0.0
RFLP Harrington	70	228	30.7
CDC Candle	67	91	73.6

1.3 Effect of inoculum age on covered smut

Objective: To investigate the effect of storage time of inoculum on covered smut infection.

CDC Candle was inoculated with *U. hordei* teliospores collected in 1997, 1998, 1999 and 2000 from field or greenhouse. The seeds were sown in the greenhouse and field to determine the level of infection. In the greenhouse, there were 4 replications/treatment (one pot = one replication) and 5 seeds/pot. The disease was scored as per cent infected plants. In the field, there were 4 replications/treatment (one row = one replication) and each row was 12 feet in length. The disease was scored as per cent infected heads as mentioned earlier.

Results indicated that all sources of inoculum had given a high level of infection on CDC Candle (Table 3). It may be concluded that three-year old inoculum gave the same level of infection as fresh inoculum.

Table 3: Effect of inoculum age on incidence of covered smut on CDC Candle in the field and greenhouse

Inoculum Age	Per cent Infected heads (Field)		Per cent infected plants (Greenhouse)
	Mean	St Dev	
Three years	33.2	7.1	81.2
Two years	21.5	4.3	91.7
One year	24.6	4.0	86.7
Fresh	24.7	5.6	75.5

1.4 Effect of inoculation time

Objective: To determine the effect of time of inoculation on the level of infection.

Preliminary results showed that the window between inoculation and planting time may be larger than previously thought as there was no difference in the percentage of infected heads from seed inoculated two months prior to planting and seed inoculated two weeks prior to planting.

This experiment was designed to establish the maximum length of time that inoculated seeds could be stored without affecting the level of infection. One hundred grams of seed of CDC Candle were inoculated with *U. hordei* on the 15th of every month starting from April 2000 through May 2001. After drying, half of the inoculated seeds were stored at 4°C and the second half at room temperature. There were 29 treatments in total and one control (uninoculated CDC Candle). The seeds were sown in the greenhouse and field to determine the level of infection.

Table 4: Effect of date of inoculations on the incidence of covered smut in the field and greenhouse

	Field		Greenhouse	
Months after	% infected heads		% infected plants	
Inoculations	Room temp.	4°C	Room temp.	4°C
check	5.9	6.2	0.0	0.0
0	41.7	42.8	70.0	75.0
0.25	44.7	44.4	100.0	57.1
0.5	43.8	39.9	70.0	50.0
1	40.7	40.1	88.9	62.5
2	40.3	44.2	71.4	37.5
3	45.4	45.1	70.0	72.7
4	38.5	43.1	45.4	66.7
5	39.0	38.8	45.4	33.3
6	40.3	42.2	66.7	60.0
7	30.2	34.3	57.1	58.3
8	32.5	38.9	53.8	70.0
9	20.1	29.0	42.8	23.1
10	14.1	41.3	26.7	54.5
11		34.9		80.0
12		35.2		50.0
13		33.0		18.2
LSD at 0.05	9.3		45.9	

In the greenhouse, there were 4 replications/treatment (one pot = one replication) and 5 seeds/pot. The disease was scored as per cent infected plants. In the field, there were 4 replications/treatment (one row = one replication) and each row was 12 feet in length.

The disease was scored as per cent infected heads as mentioned earlier. Statistical analysis was done using SAS program.

There was no disease on the uninoculated check in the greenhouse, however, 5.9 – 6.2 % infected heads were observed in the field (Table 4). This was probably because the same seed drill was used for the whole experiment in the field and some teliospores may have stuck to the drill and infected the uninoculated CDC Candle, which is very susceptible to covered smut. It is clear from the field data that the inoculated seeds can be kept for six months at room temperature and for a year at 4°C as the % infected heads were statistically same. The same results were found in the greenhouse and large differences between some of the treatments may be due to the low number of plants screened or emerging in the greenhouse.

The results clearly indicate that the inoculated seeds can be kept for 6-12 months without affecting the covered smut incidence. The implication of these findings is that when a population is screened in the greenhouse or field, the remaining inoculated seeds can be used 3 months later to confirm the resistance of putative resistant lines. This will save both time and labour.

1.5 Effect of dehulling on covered smut

Objective: To see whether the hull of the barley seed plays any role in imparting resistance to covered smut as most of the resistant cultivars are hulled.

Four hulled cultivars viz. Q21861, CDC Dolly, TR244 and RFLP Harrington were selected for this experiment. Seeds were soaked in tap water for 1-2 hours and then dehulling was done manually. About 600 seeds of each cultivar were dehulled and were inoculated with *U. hordei*. This experiment was conducted in the field and greenhouse.

In the greenhouse, there were three treatments: Dehulled inoculated; Dehulled uninoculated and Hulled inoculated. There were five replications of each cultivar for each treatment. Five seeds were sown in each pot. In field, there were only two treatments viz. Dehulled inoculated and hulled inoculated and 4 replications/treatment (one row = one replication). Statistical analysis was done using SAS program.

In the field, all dehulled inoculated seed showed numerically higher levels of infection than hulled seed, however, this was statistically significant only in the susceptible cultivar RFLP Harrington (Table 5.1).

In the greenhouse, dehulled uninoculated seeds did not develop infection, as expected (Table 5.2). Dehulled seeds had good germination indicating that dehulling did not affect the seed germination. Unlike in the field, no infected plants were observed among resistant cultivars in the hulled inoculated treatment, this might be due to the fact that only a few plants could be screened in the greenhouse. The significant difference between infected plants from hulled and dehulled inoculated seeds in CDC Dolly may also be due to the small amount of data that could be collected in the greenhouse.

Table 5.1: Dehulling experiment in the field; the data is in % infected heads

Treatment	Cultivars				Mean
	Q21861	TR244	CDC Dolly	RFLP Harrington	
Dehulled	0.5	0.08	4.3	44.8	12.4
Hulled	0.1	0.00	2.6	17.1	4.9
Mean	0.3	0.04	3.5	31.0	8.7

LSD for treatments - 2.80
LSD for cultivars - 3.97
LSD for treatment * cultivars - 5.60

As in the field, removing the hull of seeds increased the percentage of infected heads/plant of resistant lines however, this was not statistically significant. Again, the level of infection can be significantly increased in susceptible lines by dehulling. The results showed that even by removing the hull of barley seeds, the resistant cultivars gave the same level of resistance in the field and greenhouse. Therefore, the hull of barley seeds in these cultivars does not play any role in imparting resistance to covered smut and resistance of these hulled lines can be used to develop hulless barley resistance to covered smut.

Table 5.2: Dehulling experiment in the greenhouse; the data is as % infected plants

Treatment	Cultivar				Mean
	Q21861	TR244	CDC Dolly	RFLP Harrington	
Deh uninoc.	0.0	0.0	0.0	0.0	0.0
Deh inoc.	0.0	8.0	18.3	63.0	22.3
Hulled inoc.	0.0	0.0	0.0	52.7	13.2
Mean	0.0	2.7	6.1	38.5	11.8

LSD for treatments - 10.0
LSD for cultivars - 11.6
LSD for treatment * cultivars - 20.1

1.5 Screening of differentials of covered smut

Objective: To study the race spectrum of *U. hordei* inoculum used in this project.

The initial inoculum (*U. hordei* teliospores) was supplied by Dr J. Menzies, Agriculture and Agri-Food Canada, Winnipeg, obtained from fields in the Winnipeg area. This was a

mixture of number of races of *U. hordei*. The inoculum was collected every year from field and greenhouse experiments and used for further studies. Ten differential lines for covered smut of barley (received from Dr John Sherwood, Montana State University, Bozeman, USA) were inoculated with *U. hordei*. In the greenhouse, there were 5 replications/differential (one pot = one replication) and in field, there were 4 replication/differential (one row = one replication).

The inoculum of *U. hordei,* we were using for inoculations, was virulent on most of the differentials except Jet and Pannier (Table 6). This indicated that it was a mixture of 2 or 3 races, most probably races 11, 6 and 2 as reported by Tapke (1945). The susceptibility of Hannchen and Plush/Lion indicates that the mixture does not have avirulence genes V1 and V6 (see section 5) and may have avirulence gene V2 which is known to be affected by "environmental factors" (Guus Bakkeren, personal communication). Trace infection on Excelsior may be due to this effect.

Table 6: Incidence of covered smut on differentials in the field and greenhouse with the mixture of isolates of *U. hordei*

Differentials	Field % infected heads		Greenhouse % infected plants	
	Mean	SD	Mean	SD
CDC Candle	46.4	16.3	70.0	20.9
Himalaya	8.0	3.0	15.0	33.5
Nepal	13.6	2.1	19.0	20.7
Lion	1.5	1.3	8.0	17.9
Jet	0.0	0.0	0.0	0.0
Pannier	0.0	0.0	0.0	0.0
Hannchen	13.2	4.2	29.3	28.9
Odessa	19.4	6.5	21.3	19.7
Trebi	10.9	1.6	42.7	13.8
Excelsior	2.3	1.2	5.0	11.2
Plush	26.8	5.8	60.0	18.1

2. Identification of sources of resistance to covered smut, development of segregating populations and the inheritance of resistance

2.1 Sources of resistance to covered smut

Many hulled and hulless genotypes from CDC, Saskatoon and AAFC, Brandon barley breeding programs were screened for their resistance/susceptibility to covered smut (Table 7). Most of these lines were tested for 2-3 years in the field and several times in the greenhouse.

Table 7 : Screening of hulled and hulless barley cultivars/lines against covered smut in field and greenhouse

Cultivar/Line	Hulled/Hulless	% infected heads in field	% infected plants in greenhouse
Q21861	+H	0.2	4.1
SM89010	+H	2.8	23.8
TR244	+H	0.5	6.0
HB328	-H	18.1	75.8
TR337	+H	4.8	36.8
CDC Gainer	-H	6.1	20.0
CDC Dolly	+H	0.9	5.5
RFLP Harrington	+H	13.1	67.8
Baronesse	+H	7.3	65.4
TR336	+H	1.2	18.0
SB93763	-H	3.2	47.0
TR246	+H	1.7	7.4
HB334	-H	1.2	45.4
Manley	+H	1.5	25.0
CDC McGwire	-H	2.5	34.7
TR640	+H	0.0	0.0
CDC Freedom	-H	22.2	38.1
HB348	-H	3.5	5.0
TR255	+H	0.0	10.5
TR251	+H	4.3	5.3
CDC Helgason	+H	0.5	0.0
CDC Candle	-H	43.8	81.9
Varieties/lines tested in field only			
CDC Sisler	+H	36.4	
AC Metcalfe	+H	5.2	
CDC Kendall	+H	12.3	
TR359	+H	6.4	
TR361	+H	1.9	
CDC Buck	+H	39.3	
CDC Alamo	-H	18.6	
CDC Bold	+H	0.8	
CDC Dawn	-H	7.6	
CDC Silky	-H	39.0	
CDC Speedy	-H	31.6	
CDC Richard	+H	19.3	
CDC Guardian	+H	3.0	
CDC Earl	+H	20.1	
CDC Fleet	+H	5.0	
AC Bacon	-H	22.4	
Falcon	-H	1.6	

We devised a rating scale (Table 8) to classify barley cultivars/lines based on their reaction to covered smut in the field and greenhouse.

Table 8: Rating scale to classify the reaction of barley cultivars/lines to covered smut

Reaction	Field (% infected heads)	Greenhouse (% infected plants)
Resistant (R)	< 1.0	< 10.0
Moderately Resistant (MR)	1.1-3.0	10.1-25.0
Moderately Susceptible (MS)	3.1-5.0	25.1-50.0
Susceptible (S)	> 5.0	> 50.0

Based on this classification, the results can be described as:

Resistant Cultivars/lines: Q21861, TR244, TR640, CDC Dolly, CDC Helgason, CDC Bold

Moderately resistant: TR246, TR336, SM89010, Manley

Moderately Susceptible: TR337, CDC McGwire, HB348, HB334, TR251, SB93763, CDC Guardian, CDC Fleet

Susceptible: HB328, CDC Gainer, RFLP Harrington, Baronesse, CDC Freedom, CDC Sisler, AC Metcalfe, CDC Kendall, CDC Buck, CDC Dawn, CDC Silky, CDC Speedy, CDC Richard, CDC Earl, AC Bacon

CDC Candle is the most susceptible cultivar.

Some cultivars were tested once or twice in the field only, therefore, their reaction needs to be confirmed in greenhouse. No cultivar was completely resistant to covered smut except TR640. However, TR640 showed susceptibility to certain isolates from Dr Guus Bakkeren, AAFC-Summerland, BC (see section 5.1). It has been reported earlier that resistance in barley to covered smut was incomplete (Person and Cherewick 1964).

2.2 Development of segregating populations

Segregating populations were developed (Table 9) by crossing resistant cultivars with susceptible/moderately susceptible hulless/hulled cultivars and using doubled haploid, single seed decent (SSD) or regular pedigree breeding methods.

The two populations viz. HB335 x TR251 and HB334 x TR251 mentioned in the Project Design # 3 were not used in this project as TR251 was not a good source of resistance to

covered smut as it gave more than 4 % infected heads (Table 7) in the field in the initial testing. However, many other populations involving R x S parents including hulless x hulled cultivars were used.

Table 9: Segregating populations used in this project

Cross #	Pedigree	Source**	Number of lines
1	**Q 21861*** X SM89010	AC	127
2	HB328 X **TR244**	AC	324
3	**CDC Dolly 6** X RFLP Harrington	SSD	116
4	TR337 X **TR244**	SSD	127
5	CDC Gainer X **TR244**	F5	167
6	SB93763 X **TR246**	SSD	102
7	HB334X **MANLEY**	SSD	167
8	CDC McGwire X **TR640**	SSD & MC	180
9	CDC McGwire X **Q21861**	SSD	120
10	CDC Candle X **Q21861**	SSD	160

*Cultivars/lines in bold are resistant to covered smut
** AC- Anther culture derived DH lines
MC- Microspore culture derived DH lines
SSD- Single seed decent method

2.3 Inheritance of resistance studies

Inheritance of resistance in the resistant cultivars has been studied by screening the segregating populations in the field for 2-3 years after inoculation with a mixture of isolates of *U. hordei* and by confirming the reaction of putative resistant lines in greenhouse trials. Inheritance studies were not proposed in this project, however, understanding the inheritance of resistance is valuable for planning crosses in breeding programs and to identify resistant genes.

In the field, seeding was done on the last week of May or the first week of June every year at the Preston Plots, University of Saskatchewan. There were 4 replications of each line (one row = one replication). Each row was 12 feet in length. The parents and check were widely distributed. In every 56 rows, there were two rows of each parent and one row of check. CDC Candle, the most susceptible cultivar, was used as a susceptible check in all the experiments.

In the greenhouse, putative resistant lines were also screened to confirm their reaction. There were three replications of each line (one pot = one replication) and five seeds were sown in each pot.

Disease Evaluations:
In the field, the level of covered smut infection was evaluated as the percentage of infected heads. Two 1 m sections were measured within each row. In each 1 m section, counts of the total number of heads and number of infected heads were conducted. The two counts were averaged together for each replication and the percentage of infected heads was calculated.

In the greenhouse, the level of covered smut infection was evaluated as the percentage of infected plants. A plant showing one or more smutted heads was considered to be infected. In each pot, the number of infected plants and total plants were counted and percentage of infected plants was calculated.

Analysis of data
A cut-off point was used to distinguish the resistant and susceptible classes in each population. This cut-off was established after reviewing the disease score of the parents and the population. A Chi-square test was used to check the fitness of segregation classes.

Results
A single major gene for resistance was found in Q21861 based on three years of screening the doubled haploid population Q21861/SM89010 in the field and two seasons of screening in the greenhouse.

Table 10: Inheritance of resistance studies in covered smut segregating populations

Cross #	Pedigree	R:S lines	Ratio with Chi-square values	No. of gene (s)
1	**Q21861*** X SM89010	67:60	1:1 (χ^2 = 0.38; P= 0.50-0.75)	Single gene
2	HB328 X **TR244**	152:172	1:1 (χ^2 =1.24; P=0.25-0.50)	Single gene
3	**CDC Dolly 6** X RFLP Harrington	23:93	1:3 (χ^2=1.65, P= 0.10-0.25)	Two recessive genes
4	TR337 X **TR244**	66:61	1:1 (χ^2 =0.20; P=0.50-0.75)	Single gene
5	CDC Gainer X **TR244**	68:99	1:1 (χ^2 =5.75; P=0.10-0.25)	Single gene
6	SB93763 X **TR246**	49:53	1:1 (χ^2=0.34, P=0.50-0.75)	Single gene
7	HB334X **MANLEY**			See text
8	CDC McGwire X **TR640**	51:49	1:1 (χ^2=0.04; P=0.84)	Single gene
9	CDC McGwire X **Q21861**	63:54	1:1 (χ^2=0.69, P=0.40)	Single gene
10	CDC Candle X **Q21861**	87:80	1:1 (χ^2=0.29; P=0.59)	Single gene

*Cultivars/lines in bold are resistant to covered smut

Similarly, a single gene for resistance has been found in TR244, TR246 and TR640 in different segregating populations (Table 10). A single dominant gene governing resistance to covered smut has been reported in many barley cultivars (Shands 1956; Wells 1958, Metcalfe 1962, Cherewick and Buchannon 1969). However, in the cross CDC Dolly/ RFLP Harrington, the resistant and susceptible lines segregated in a 1:3 ratio indicating two recessive genes for resistance in CDC Dolly.

In HB334/Manley population, both the parents were moderately susceptible/resistant to covered smut (gave <2 % infected heads in the field), however, we found transgressive segregation in the population. Many lines have better resistance than the both parents and many others were highly susceptible indicating that gene (s) for resistance present in HB334 and Manley were different. The lines giving complete or better resistance may have genes from both the parents indicating additive gene effect. These lines can be used as sources of resistance or may be released as cultivars if they are agronomically good.

2.4 Screening of SFBYT and EPBYT entries

This experiment was conducted to assist the Saskatchewan Feed Barley and Eastern Prairie Barley programs.

In Summer 2001, 263 entries from the Standard Feed Barley Yield Trials (SFBYT) and 30 entries from Eastern Prairies Barley Yield Trials (EPBYT) were inoculated with *U. hordei* and planted in the field. There was one replication/entry consisting of a 12-foot row. Among SFBYT entries, 50 % (130 entries) showed less than 5 % infected heads, 12 entries were disease free and 47 entries showed less than 1 % infected heads indicating good sources of resistance to covered smut are available among elite breeding lines. Twelve EPBYT entries showed less than 5 % infected heads.

In Summer 2002, 44 two-rowed Co-op, 22 Hulless Barley Co-op, 30 EPBYT and 271 SFBYT entries were screened in the field using mixture of isolates of *U. hordei*. Among two-rowed Co-op entries, 31(70 %) showed less than 5 % infected heads and 16 % showed good level (<1 % infected heads) of resistance to covered smut. Only one HB Co-op entry showed less than 1 % infected heads confirming that hulless barley is very susceptible to this disease and there is need to incorporate covered smut resistance into hulless barley. Fifty per cent of the EPBYT entries were less than 5 % infected heads and 10 % were showing less than 1 % infected heads. Among 271 SFBYT entries, 39 % showed less than 5 % infected heads and 23 entries (8%) showed less than 1 % infected heads.

It is desirable to screen the elite breeding material against covered smut as some lines are extremely susceptible and should not be released for cultivation as this may increase the covered smut incidence and severity.

3. Identification and development of molecular markers

This disease is ideally suited for MAS as the disease is economically important, the resistance is primarily due to a single gene, and phenotyping is expensive and unreliable. Breeding for resistance to covered smut is not routinely performed because the traditional screening is time consuming (requiring a complete generation) and thus requires considerable resources. In addition, artificial inoculation is inconsistent and we have to screen the putative resistant lines again and again to confirm that there is no escape.

3.1 Identification of molecular markers

3.1.1 RAPD Analysis

The Q/SM population was used to develop molecular markers with RAPD primers using bulked segregant analysis (BSA). Of the 440 10-mer random primers tested, primer OPJ10 resulted in a reproducible bright polymorphic band of ~450 bp present in the susceptible bulk, the susceptible parent (SM89010) and each individual of the susceptible bulk but not in the resistant bulk, resistant parent (Q21861) and resistant individuals (Fig.1).

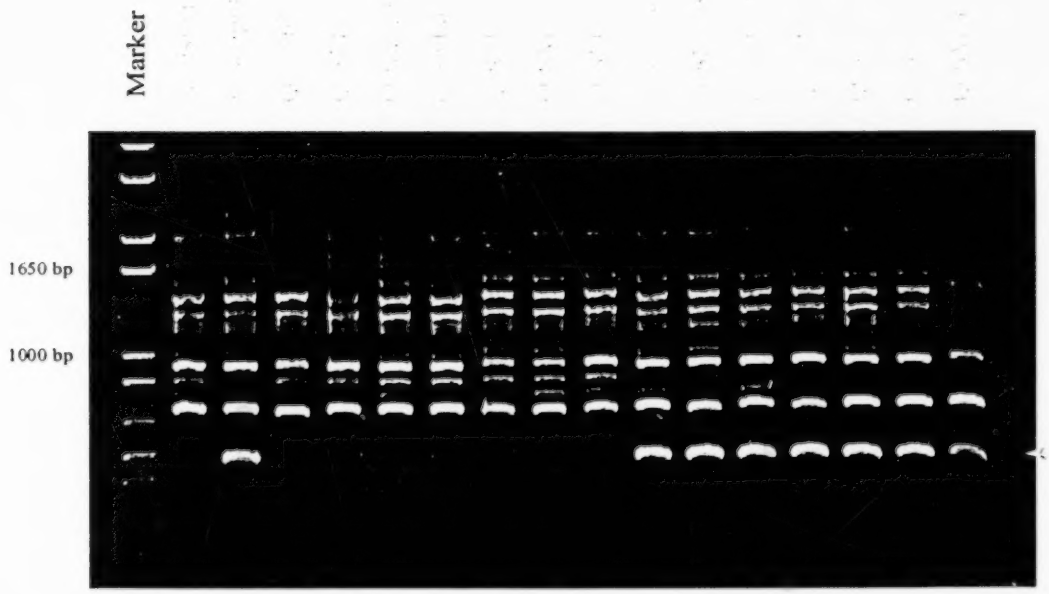

Fig.1 DNA amplification from the covered smut resistant bulk, susceptible bulk, resistant parent (Q21861), individuals of the resistant bulk, susceptible parent (SM89010) and individuals of the susceptible bulk with RAPD OPJ 10 primer (arrow indicates polymorphic band).

The entire population was screened with this primer. Seven of 127 individuals were recombinants between the marker and the resistance gene suggesting linkage in repulsion to covered smut resistance of 5.5 cM (Table 11).

Table 11: The relationship between the OPJ10 RAPD polymorphism and covered smut rating

		Disease Rating		
		Resistant	Susceptible	Total
DNA Polymorphism Pattern	Resistant	65	5	70
	Susceptible	2	55	57
	Total	67	60	127

3.1.2 AFLP Analysis

Q21861/SM89010 population

All of the AFLP primer pairs screened (82) produced scoreable banding patterns. However, only one primer pair E-AAA/M-CCC produced a polymorphic band of 265 bp in the resistant bulk, resistant parent and all individuals constituting the resistant bulk. This band was not present in the susceptible bulk, susceptible parent or any of the susceptible individuals. The entire population was screened with this primer pair and there were 11 recombinants between the marker and covered smut resistance, suggesting a linkage of this marker in coupling with the covered smut resistance in Q21861.

CDC Dolly/RFLP Harrington

Fourteen primer-pair combinations were used to screen the bulks and parents of this population. Primer-pairs E-AAC/M-CTC, E-AAG/M-CAC, E-AAC/M-CAA, E-AAC/M-CAG, E-AAC/M-CTG produced polymorphic bands between the bulks and parents.
Primer-pair E-AAC/M-CTC produced a polymorphic band of ~390 bp in the susceptible bulk, susceptible parent and all the individuals constituting the susceptible bulk whereas, primer-pair E-AAG/M-CAC produced a polymorphic band of ~450 bp in CDC Dolly and resistant bulk and all the individuals constituting the resistant bulk. Twenty-five resistant and 25 susceptible lines were screened with these two primer-pairs. The band produced by primer-pair E-AAC/M-CTC was positive in 42 out of 50 lines tested suggesting a linkage of 16 cM whereas; primer-pair E-AAG/M-CAC was positive in 39 out 50 lines. Six lines that gave false +ve/-ve results were in common with both the putative markers.

TR244/HB328

Fourteen primer-pair combinations were used to screen the bulks and parents. Primer-pairs E-AAC/M-CAC, E-ACG/M-CTA, E-AAC/M-CAG and E-AAC/M-CTG produced polymorphic bands between the bulks and parents. Primer-pair E-AAC/M-CAC was

used to screen the individuals constituting the bulks and 30 more lines from this population. This primer-pair produced a polymorphic band of 430 bp present in the susceptible bulk, the susceptible parent and all the individuals constituting the susceptible bulk, however, in the additional 30 lines it produced 10 recombinants. Therefore, this was loosely linked to covered smut resistance/susceptibility.

3.1.3 Screening with SSR primers

Simple sequence repeats (SSRs) are extremely common throughout the eukaryotic genome and are highly polymorphic in length (Tautz 1989). A representative set of barley SSR primers (Macaulay et al. 2001) used to determine the linkage of microsatellite markers with resistance to covered smut in many segregating populations using BSA. Nineteen primer-pairs from core SSR primer set were used to screen the bulks and parents of three populations i.e. Q21861/SM89010, HB328/TR244 and RFLP Harrington/CDC Dolly. Many primers showed polymorphic bands between the bulks and the parents. However, we could not find any microsatellite marker linked to covered smut resistance in these populations.

3.1.4 Screening of the TR244/HB328 population with ISSR primers

Inter simple sequence repeat PCR (ISSR-PCR) exploits the abundance of SSRs to generate complex banding profiles that vary both within and between species. In contrast to the SSR-PCR method that amplifies with primers located on the single copy DNA, microsatellite anchored primers that anneal to an SSR region can amplify regions between adjacent SSRs.

One hundred SSR primers from the Biotechnology Lab, UBC (primer set # 9) were tested for PCR amplification against resistant and susceptible bulks of this population. We initially examined the optimal conditions for detecting ISSRs, as there was no published report in barley. The effect of template concentration, formamide and Triton X-100 were examined. The template concentration and Triton X-100 did not have much effect whereas formamide had a significant effect on banding pattern.

Amplifications were carried out in 2.0 mM $MgCl_2$, 100 µm of each dNTP, 2 % formamide, 200 nM primer, 1 unit of *Taq* polymerase and 50 ng of genomic DNA per 25 µl reaction. Initial denaturation was for 5 min at 94°C, followed by 45 cycles of 30s at 94°C, 45s at 50°C, 2 min at 72°C and a final 10 min extension at 72°C. The amplified products were electrophoresed on 1.5 % agarose gels and detected by staining with ethidium bromide.

Of the 100 primers 57 gave strong bands among the bulks. Primer UBC 859 produced a bright reproducible band of 1100 bp in the resistant parent, resistant bulk and 4 individuals of the resistant bulk and was absent in the susceptible parent, susceptible bulk and all individuals of the susceptible bulk. Then 66 lines were tested with this primer and

there were 21 recombinants suggesting that it was only loosely linked to the covered smut resistance of TR244.

It was decided to use primer-pair combinations as reported by Cekic *et al.* (2001) on *Fragaria* spp. Ten primers viz. UBC 810, 824, 828, 834, 843, 848, 855, 859, 876 and 899 were selected based on clear amplicons produced by them on bulks. All possible pair-wise combinations of primers were used on bulks. The primer concentration was 100 nM of each and all other conditions were same. Many primer-pairs produced polymorphic bands between the bulks, however, 4 primer-pairs (843:848, 843:855, 848:899, 855:876) gave reproducible polymorphic bands between the bulks and parents. When these primer-pairs were tested on individuals of resistant and susceptible bulks, they were positive in 8 or 9 individuals. However, among 66 lines tested, there were 18 to 23 recombinants. Interestingly 13 recombinant lines were common for all these primer-pairs and UBC 859. There may be two possibilities:

1. All these primers are targeting the same genomic region and giving the same recombinants.
2. As we are using mixture of isolates for screening, the presence of many isolates may be distorting the segregation ratio.

Many novel polymorphisms were generated by the combined use of two ISSR primers as opposed to the same primers used individually. The pair-wise combinations produced new and distinct band profiles. Interestingly all these putative markers were co-dominant however, this technique failed to produce useful markers.

There is a need to identify molecular markers linked to the covered smut resistance of TR244, CDC Dolly and other sources. These markers may be used to pyramid different covered smut resistance genes in the elite breeding lines.

3.2 Development of SCAR marker

A RAPD marker $OPJ10^{450}$ tightly linked (5.5 cM) with covered the smut resistance of Q21861 was identified using a doubled haploid population of Q21861 X SM8910. However, the RAPD technique is sensitive to reaction conditions, which results in poor reproducibility. To overcome this problem, longer primers can be developed from RAPD fragments (Paran and Michelmore 1993) and these longer primers generate a sequence characterized amplified region (SCAR). SCARs are advantageous over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions and they can be potentially converted into allele specific markers.

The polymorphic band produced by the primer OPJ10 was excised, cloned and sequenced. Two primers were developed from the amplified sequence but the same sized band was amplified in both the susceptible and resistant parent. The band present in the resistant and susceptible genotypes was cloned and sequenced. Several primers complementary to the ends of the fragment designed based on single base difference between the two sequences. These were synthesized and tested in various combinations until a single band was produced in the resistant genotype only. The primer pair ULR1-F

(GATAAGGATGTTCCGCC) and ULR-R (CCCGAGGTCCAAAATCAG) yielded a major band of expected size (442 bp) in the resistant genotypes and was absent in the susceptible genotypes (Fig. 2). The polymorphic banding pattern was reproducible and the entire population was screened with these SCAR primers. The SCAR marker was found to co-segregate fully in repulsion with the OPJ10 RAPD marker.

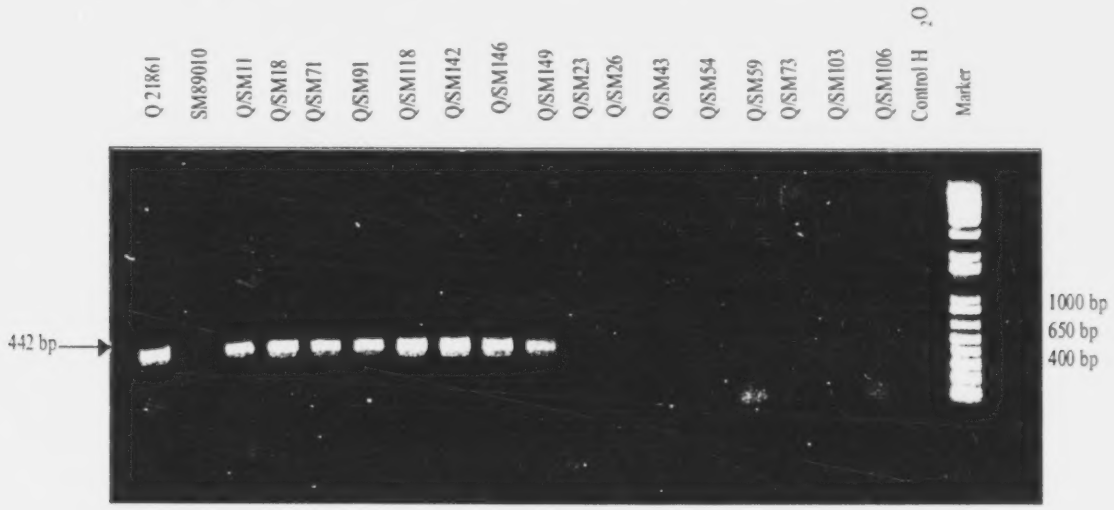

Fig. 2 Amplification product (442 bp) obtained with SCAR primers ULR1-F and ULR-R; present in resistant parent (Q21861) and resistant lines and absent in susceptible parent (SM89010) and susceptible lines.

The parents of other populations segregating for covered smut resistance and of many mapping populations of barley were screened with these SCAR primers. The 442 bp band was amplified in only Q21861 and the line TR640 (also resistant to covered smut) and absent in all other parents.

4. Mapping and validation of molecular markers for MAS

Mapping of a molecular marker is important as it identifies the location of a marker on a particular chromosome. This allows other marker loci that might be linked to the trait of interest to be identified and these may also be useful as markers for the trait and could be used in marker-assisted selection.

4.1 Mapping of the RAPD marker OPJ10^{450}

The SCAR marker, developed from the RAPD marker OPJ10^{450}, was present only in Q21861 and TR640 and absent in all other parents of the covered smut segregating populations and also was not produced in any of the barley mapping populations. Therefore, we could not map the SCAR marker and it was decided to screen the parents of mapping populations with the random primer OPJ10 to see if the RAPD marker could be mapped.

A new stock of the primer OPJ10 was used to screen the parents of four mapping populations (Harrington/TR306, Steptoe/Morex, Igri/Franka, Proctor/Nudinka) and also Q21861 and SM89010 using the same PCR reaction conditions as reported earlier (Ardiel *et al.* 2001). There was no polymorphism in any of the parents even in Q21861 and SM89010, where it had been detected originally. The band of interest (~450 bp) was present in all the parents. To obtain a polymorphism between Q21861 and SM89010, different concentration of the primer (1µl to 6µl) and $MgCl_2$ (0.5µl to 4.0 µl) were tried. The different concentrations of primer gave the same results, however a $MgCl_2$ conc. of 2.5 µl or higher/reaction mixture resulted in the reproducible original polymorphic band i.e. a bright band of ~450 bp present in SM89010 and a faint band of same size in Q21861. A $MgCl_2$ conc. of 3.0 µl/reaction mixture (6.0 mM) was used for further experiments. The resistant and susceptible bulks from the Q/SM population and the individual constituents of the bulks were screened to confirm the polymorphism. The polymorphic band was reproduced as reported earlier and then the parents of mapping populations viz. TR306/Harrington, Steptoe/Morex, Igri/Franka and Proctor/Nudinka were screened. The polymorphic band present in SM89010 was also present in TR306 and absent in Harrington but there was no polymorphism between the parents of other mapping populations.

The polymorphic band between TR306 and Harrington was reproducible and the whole mapping population (149 doubled haploid lines) was screened with the primer OPJ10. This data was compared with the mapping data of this population and was close to marker Act8A on the short arm of chromosome 5 (homoeologous chromosome 1H).

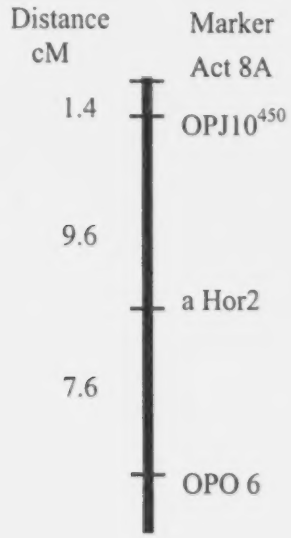

Fig. 3: A linkage map showing RAPD marker $OPJ10^{450}$ and other markers on the short arm of chromosome 5 (1H) of mapping population Harrington/TR306. Distance reported in Kosambi centiMorgans units.

MAPMAKER/EXP V3.0 (Lander *et al.*, 1987) was used to determine its linkage with other markers on the short arm of chromosome 5 and we obtained this linkage map (Fig. 3).

4.1.1 Screening with the aHor2 marker

The aHor2 locus encoding B hordein (a seed storage protein in barley) is also located on the short arm of chromosome 5 (Fig. 3). It is a co-dominant PCR-based marker producing an 800 bp band in TR306 and 1100 bp size band in Harrington. As it is near to the RAPD marker $OPJ10^{450}$, the parents of covered smut segregating populations were screened with this marker.

PCR reactions were performed in a total volume of 25 µl, containing 200 µm each of dATP, dTTP, dCTP and dGTP (Gibco BRL, Bethesda, USA), 400nM of each primer (KV1 and KV9), 50 ng of genomic DNA, 1.5mM $MgCl_2$, 1 unit of Taq DNA polymerase (CMG Lab Taq) and 1X PCR buffer (Gibco BRL). Amplification was performed with an Amplitron® II thermocycler (Barnstead/Thermolyne, USA) and consisted of an initial denaturation step at 94° C for 6 min, followed by 35 cycles as follows; a denaturation step at 94°C for 30 sec, an annealing step at 63° C for 1 min and an extension step at 72° C for 1 min; ending with an extension period of 72° C for 10 min. All amplification products were separated on a 1 % agarose, 0.5 X TBE gel, stained with ethidium bromide and viewed under ultra-violet light.

The aHor2 marker was polymorphic between parents of many populations that were segregating for covered smut resistance viz. Q21861 and SM89010, Q21861 and CDC Candle, CDC Dolly and RFLP Harrington, Manley and HB334 (Table 12). No band was produced in HB328, CDC Gainer, SB93763 and HB348 after several attempts and using DNA of these cultivars from different sources.

Table 12: Screening of parents of segregating populations with aHor2 marker (a PCR-based co-dominant marker producing bands at 800 bp and 1100 bp)

Cultivar/Line	aHor2 Marker	
	800 bp band	1100 bp band
Q21861		Yes
SM89010	Yes	
CDC Candle	Yes	
CDC Dolly	Yes	
RFLP Harrington		Yes
TR244	Yes	
HB328	-	-
TR337	Yes	
CDC Gainer	-	-
TR336		Yes
Baronesse		Yes
TR246		Yes

SB93763	-	-
TR640		Yes
CDC McGwire		Yes
CDC Freedom		Yes
TR255	Yes	
Manley	Yes	
HB334		Yes
TR251		Yes
HB348	-	-
TR306	Yes	
Harrington		Yes

4.1.1.1 Screening of the Q/SM population with aHor2

As this marker produced a 1100 bp band in Q21861 and an 800 bp band in SM89010, the resistant and susceptible bulks and their constituents were screened with this marker to determine its linkage with covered smut resistance/susceptibility. The marker produced a 1100 bp band in the resistant bulk and all the individuals constituting the resistant bulk and produced the 800 bp band in the susceptible bulk and individuals constituting the susceptible bulk (Fig. 4). All lines from this population were screened with this marker and there were 12 recombinants between the marker and covered smut reaction.

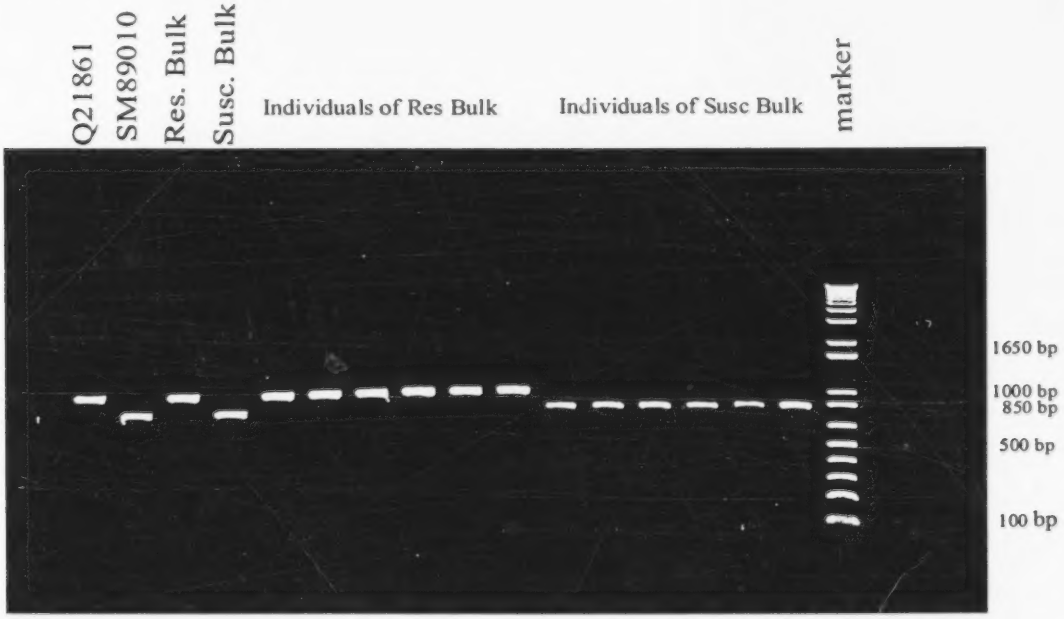

Fig. 4 Amplification products obtained with aHor 2 primers KV1 and KV9; 1100bp band is present in Q21861, resistant bulk and individuals of resistant bulk and 800bp band is present in SM89010, susceptible bulk and individuals of susceptible bulk.

The aHor2 marker was found to be closely linked to covered smut resistance in Q21861 line. It might be better to use this marker in MAS because it is a co-dominant marker and there is no chance of false negatives. It has been reported that Hor2 is also linked to powdery mildew resistance (Jensen *et al.* 1980), scald resistance (Gravin *et al.* 2001) and Russian Wheat Aphid resistance (Nieto-Lopez and Blake, 1994) in barley.

4.1.1.2 Screening of other populations with aHor2

As aHor2 was polymorphic between CDC Dolly and RFLP Harrington, Manley and HB334, and TR244 and HB328, the most resistant and susceptible lines from these three populations were tested to determine if aHor2 was linked with the covered smut resistance of these cultivars. In all these populations, aHor2 was not linked to the resistance. Therefore, resistance in these cultivars may be different from that of Q21861. Thus there is a need to find markers linked to these sources of resistance so that such markers could be used to pyramid many covered smut resistance genes in elite material.

4.1.2 Screening of the Q/SM population with OPO6

Random primer OPO6 (CCACGGGAAG) produced a polymorphism on the HT mapping population which was mapped to the short arm of chromosome 5 by Eckstein and Scoles, 1993 (http://barleygenomics.wsu.edu/databases/databases.html). As it is near to RAPD marker $OPJ10^{450}$, it was decided to determine its linkage with covered smut resistance of Q21861 in the Q/SM population. The PCR reaction was performed in a total volume of 25 µl, containing 100 µm each of dATP, dTTP, dCTP and dGTP (Gibco BRL, Bethesda, USA), 600nM of primer, 100 ng of genomic DNA, 2.0 mM $MgCl_2$, 1 unit of Taq DNA polymerase (CMG Lab Taq) and 1X PCR buffer (Gibco BRL). Amplification was performed with an Amplitron® II thermocycler (Barnstead/Thermolyne, USA) and consisted of an initial denaturation step at 94° C for 3 min, followed by 35 cycles as follows; a denaturation step at 94°C for 1 min, an annealing step at 37° C for 1 min and an extension step at 72° C for 1.5 min; ending with an extension period of 72° C for 10 min. All amplification products were separated on a 1 % agarose, 0.5 X TBE gel, stained with ethidium bromide and viewed under ultra-violet light.

A bright band of ~ 780 bp in size was present in Q21861, the resistant bulk and all the individuals of resistant bulk and a faint band of same size was present in SM89010, the susceptible bulk and all the susceptible individuals (Fig. 5). The whole population was screened with this primer and there were 14 recombinants between this marker and covered smut resistance of Q21861.

4.1.3 Screening of Q/SM population with Act 8A

The Act 8A marker (RFLP) previously mapped to the distal end of the short arm of chromosome 5(1H) in the HT population was very close to RAPD marker $OPJ10_{450}$. However, this marker was not polymorphic between Q21861 and SM89010, therefore linkage to the covered smut resistance in Q21861 could not be determined.

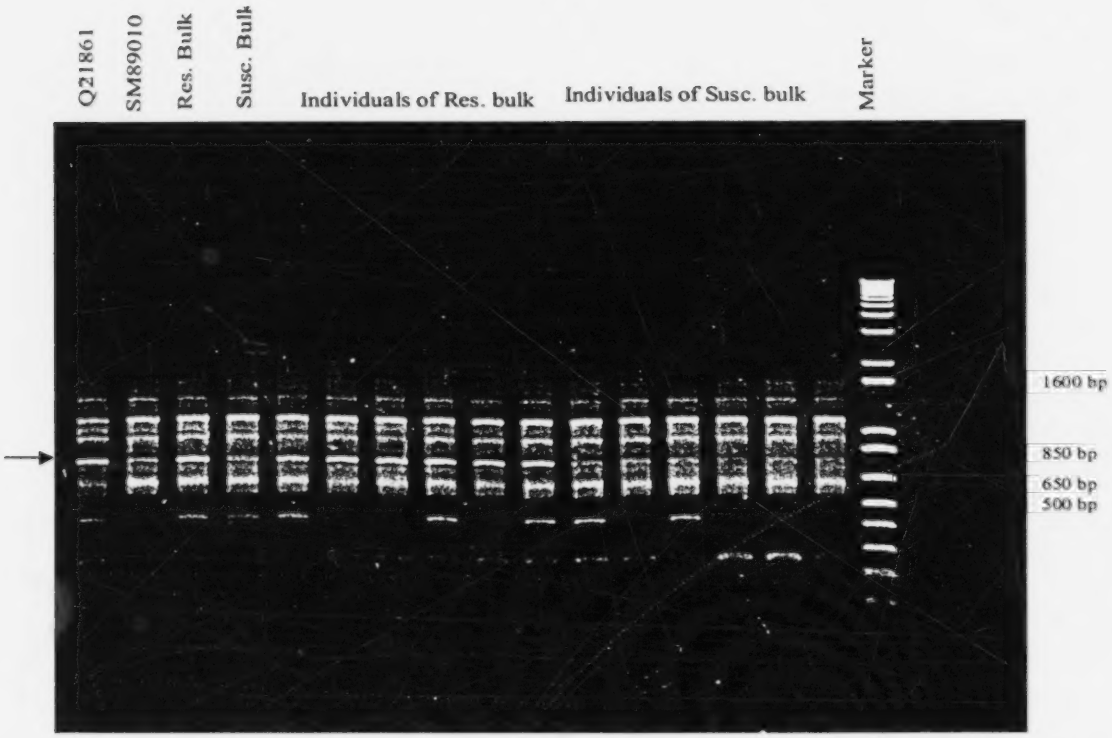

Fig. 5 Amplification products obtained with primer $OPO6_{780}$; a bright ~780bp band is present in Q21861, resistant bulk and individuals of resistant bulk and a faint band of the same size is present in SM89010, susceptible bulk and individuals of susceptible bulk.

4.1.4 Reviewing phenotypic and marker data

In reviewing the covered smut reaction and marker data from the Q/SM population, the marker patterns for three markers ($OPJ10^{450}$/UhR 450, aHor 2, $OPO6^{780}$) on seven lines was the opposite of that expected based on the disease reaction ascribed to those lines (Appendix 1). A review of the data for these seven lines (Table 13) failed to indicate any reason to suggest either that lines Q/SM 12 and 39 were not correctly scored as resistant or that Q/SM 37 was not correctly scored as susceptible. However, the four remaining lines, while categorized as susceptible may have been misclassified.

Lines Q/SM 28 and 141 displayed good resistance in the field but had been rated as susceptible in the greenhouse based on a single screening. On the other hand, no greenhouse data had been collected for lines Q/SM 46 and 148 and they were below the cut-off point (2% infected heads in the field) for covered smut reaction in 1998 and just above the cut-off point in 1999. As a result of this review of the data, the four lines Q/SM 28,46,141 and 148 were reclassified as resistant rather than susceptible. This change leads to an adjustment in the linkage values of all markers to the covered smut resistance locus (Fig. 6).

Table 13: Covered smut reaction of 7 lines that appeared to be recombinants between the resistance and all markers

Line #	% infected heads in field			% infected plants in greenhouse	
	1998	1999	2000	Spring 2000	Summer 2000
Q/SM 12	0.4	0.4	0.2	0.0	0.0
Q/SM 37	10.2	12.3	NT*	NT	NT
Q/SM 39	0.5	0.1	0.0	0.0	0.0
Q/SM 28	1.6	1.1	0.6	20.0	NT
Q/SM 46	0.2	2.1	NT	NT	NT
Q/SM 141	0.1	0.8	0.0	18.2	NT
Q/SM 148	0.4	2.1	NT	NT	NT

*NT- Not tested

4.1.5 Designation of covered smut resistance gene and its linkage to molecular markers

The covered smut resistance gene in Q21861designated as *Ruh q* based on the guidelines for nomenclature to resistance genes in barley (BGN 11:1-16). MAPMAKER/EXP V3.0 was used to calculate the linkage of the markers UhR 450, aHor2, $OPO6^{780}$ and E-$AAA/M\text{-}CCC^{265}$ with the covered smut resistance gene (Fig. 6).

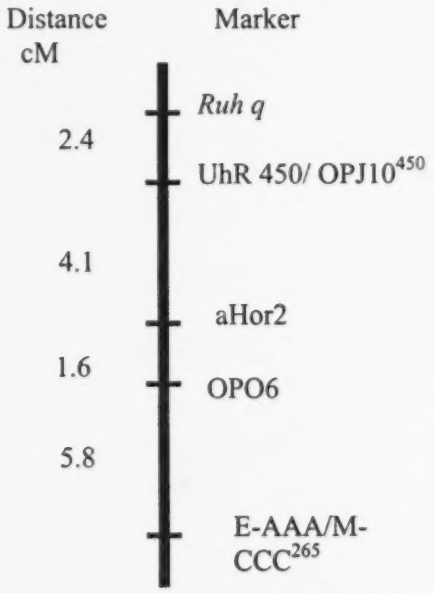

Fig. 6 A linkage map for the markers UhR 450, aHor2, OPO6, E-$AAA/M\text{-}CCC^{265}$ and *Ruh q* (covered smut resistance gene in Q21861) gene associated with covered smut resistance in the Q21861 X SM89010 population. Distances reported in Kosambi centiMorgans units.

The map shows that the SCAR marker is tightly linked to *Ruh q*; aHor2 is 6.5 cM away followed by OPO6 (8.1 cM). The AFLP marker E-AAA/M-CCC^{265} is 13.9 cM from the covered smut resistance gene.

4.2 Validation of molecular markers

The validation process is defined as the testing of an allele for its effect in genetic backgrounds other than the original mapping population (Barr et al. 2000). This is required to assess the usefulness of published reports of linked markers developed using genetic material divergent from that in target breeding programs.

We have done the validation of three markers (UhR 450, aHor 2 and OPO6^{780}) on three covered smut segregating hulless x hulled populations viz. CDC Candle/Q21861, CDC McGwire/TR640 and CDC McGwire/Q21861. The markers UhR 450 and OPO6^{780} are polymorphic between the parents of all the three populations whereas aHor 2 is polymorphic only between Q21861 and CDC Candle. UhR 450 and OPO6^{780} are dominant markers and lines that failed to amplify with these markers were tested again to confirm their reaction.

CDC Candle/Q21861 population

One hundred and sixty seven lines and the parents were screened with all the three markers. UhR 450 produced a 450bp band in Q21861 and 89 lines and the band was absent in CDC Candle and 78 lines (Appendix 2). There were 22 out of 167 lines where the marker genotype and the phenotype for resistance gene (*Ruh q*) in Q21861 line in this population did not agree suggesting a linkage distance of 13.17 cM (Table 14).

Table 14: The relationship between genotype by the molecular markers and phenotype for covered smut reaction in CDC Candle/Q21861 population

Marker	DNA polymorphism Pattern	Disease Rating		Total
		Resistant	Susceptible	
UhR 450	Resistant	77	12	89
	Susceptible	10	68	78
	Total	87	80	167
aHor 2	Resistant	73	12	85
	Susceptible	14	68	82
	Total	87	80	167
OPO6^{780}	Resistant	74	13	87
	Susceptible	13	67	80
	Total	87	80	167

aHor 2 produced an 1100bp band in Q21861 and 85 lines and an 800bp in CDC Candle and 82 lines. Twenty six of 167 individuals were recombinants between aHor 2 marker and *Ruh q*, suggesting a linkage distance of 15.56 cM. The RAPD marker $OPO6^{780}$ produced a 780bp bright band in Q21861 and 87 lines and the band of was absent in CDC Candle and 80 lines. This marker was also placed 15.56 cM away from *Ruh q* as there were 26 lines where the genotype and phenotype did not agree.

CDC McGwire/TR640 population

One hundred lines were selected from this population to screen with these markers. These lines included microspore culture derived doubled haploid lines and SSD lines. The markers UhR 450 and $OPO6^{780}$ were positive in 49 lines and negative in 51 lines of this population. The linkage distance between covered smut resistance gene in TR640 and UhR 450 was 20 cM whereas for $OPO6_{780}$, it was 28 cM (Table 15).

Table 15: The relationship between genotype by the molecular markers and phenotype for covered smut reaction in CDC McGwire/Q21861 population

Marker	DNA polymorphism Pattern	Disease Rating		
		Resistant	Susceptible	Total
UhR 450	Resistant	56	10	66
	Susceptible	7	44	51
	Total	63	54	117
$OPO6^{780}$	Resistant	55	12	67
	Susceptible	8	42	50
	Total	63	54	117

CDC McGwire/Q21861 population

To screen this population with the SCAR marker UhR 450, leaf disc supernatant DNA template was used. DNA templates were prepared by using the Alkali Treatment Protocol (modified from Klimyuk *et al.* 1993). PCR reaction and cycling conditions were standardized which were as follows:

PCR reaction was performed in a total volume of 25 µl, containing 100 µm each of dATP, dTTP, dCTP and dGTP (Gibco BRL, Bethesda, USA), 200nM of each primer, genomic DNA (5 µl of DNA prep.), 4.0 mM $MgCl_2$, 1 unit of Taq DNA polymerase (CMG Lab Taq) and 1X PCR buffer (Gibco BRL). Amplification was performed with an Amplitron® II thermocycler (Barnstead/Thermolyne, USA) and consisted of an initial denaturation step at 94° C for 3 min, followed by 37 cycles as follows; a denaturation step at 94°C for 1 min, an annealing step at 62° C for 1 min and an extension step at 72° C for 1.5 min; ending with an extension period of 72° C for 10 min.

We had to standardize the PCR reaction and cycling conditions as the earlier protocol (for DNA template prepared by CTAB method- Ardiel et al. 2002) did not worked. To have a good banding pattern with the leaf disc template DNA, the annealing temp. was decreased to 62°C (from 64° C) and the $MgCl_2$ concentration increased to 4.0 mM (from 2.0 mM) and the number of PCR cycles to 37 (from 35 cycles). The electrophoresis was done in a 96 well gel-apparatus system. The band of the expected size (450 bp) was present in Q21861 and absent in CDC McGwire (Fig. 7) and present in 66 lines out of 117 lines. The linkage distance between UhR 450 and *Ruh q* was worked out to be 13.67 cM (Table 16). $OPO6^{780}$ produced a band of expected size in Q21861 and 67 lines. There were 19 recombinants between the marker and *Ruh q*, suggesting a linkage distance of 16.23 cM.

16: The relationship between genotype by the molecular markers and phenotype for covered smut reaction in CDC McGwire/TR640 population

Marker	DNA polymorphism Pattern	Disease Rating		
		Resistant	Susceptible	Total
UhR 450	Resistant	40	9	49
	Susceptible	11	40	51
	Total	51	49	100
$OPO6^{780}$	Resistant	36	13	49
	Susceptible	15	36	51
	Total	51	49	100

The key limitation in the MAS is the cost and time for DNA extraction (Barr et al. 2000). Rapid DNA isolation systems are available (Klimyuk et al. 1993, Wang et al. 1993) and they are reported to be reliable for MAS studies (Gu et al. 1995, Raman and Read 1999). We found that the SCAR marker was robust and worked well with the rapid leaf-disc template preparation method. Reproducibility was the same as with the good quality of DNA, however, we had to modify some PCR reaction and cycling conditions to get good results. The templates can be re-used further for other PCR based markers of interest in the same cross and can be stored for months. We found that the templates stored for a year at -20°C amplified desired PCR products. aHor 2 and $OPO6^{780}$ were also amenable to leaf-disc template method. This method is highly efficient with low cost of template preparation and is routinely used coupled with electrophoresis of 96 PCR samples at once in MAS program in our lab.

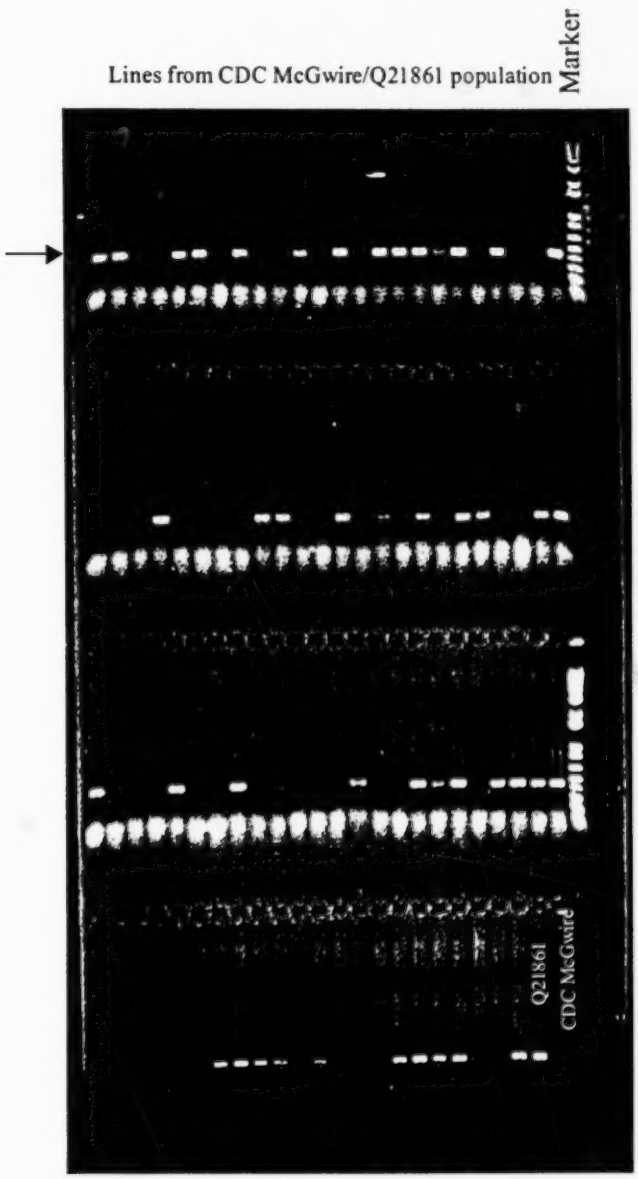

Fig. 7 Amplification product (450 bp) obtained with SCAR primers ULR1-F and ULR-R using leaf-disc template; present in resistant parent (Q21861) and resistant lines, and absent in susceptible parent (CDC McGwire) and susceptible lines.

In conclusion, we found that markers developed from a hulled x hulled population (Q/SM) can be used for selecting resistant hulless barley. All three markers were linked to covered smut resistance in the hulless populations, although aHor 2 was only polymorphic in one population.

5. Additional Experiments:

Some additional experiments have been undertaken that were not proposed originally but which developed in response to questions that arose as the work continued. These were a study of host-pathogen interactions; a study to determine whether the gene(s) present in resistant cultivars are the same or not and a plan to introgress both loose smut and covered smut resistance into the hulless cultivar, CDC McGwire using molecular markers for both diseases.

5.1 Evaluation of the genotypes of several barley lines with respect to their resistance or susceptibility to several covered smut races/isolates harboring defined avirulence genes

This experiment was conducted in collaboration with Dr Guus Bakkeren, Pacific Agri-Food Research Centre (AAFC), Summerland, BC.

Materials and Methods: Twenty four barley cultivars/lines were selected for this experiment viz. Q21861, SM89010, TR244, HB328, CDC Dolly, RFLP Harrington, TR246, SB93763, Manley, HB334, CDC Gainer, TR337, TR640, TR255, CDC McGwire, CDC Freedom, HB348, TR251, Baronesse, TR336, CDC Candle, CDC Helgason, HB353 and TR306.

Four differentials were included viz. Hannchen (harbouring at least *UhR1*), Excelsior (harbouring at least *UhR2*), Plush (harbouring at least *UhR6*) and Odessa (universal susceptible to all races).

We obtained 6 isolates of *U. hordei* from Dr Bakkeren having known avirulence/virulence genes:

Uh1	v1v1v2v2v6v6	-virulent on Hannchen, Excelsior and Plush
Uh2	V1V1v2v2v6v6	-avirulent on Hannchen, virulent on Excelsior and Plush
Uh3	v1v1V2V2v6v6	-avirulent on Excelsior, virulent on Hannchen and Plush
Uh4	v1v1V2V2V6V6	-avirulent on Excelsior and Plush, virulent on Hannchen
Uh5	V1V1V2V2V6V6	-avirulent on Hannchen, Excelsior and Plush
Uh6	v1v1v2v2V6v6	-avirulent on Plush, virulent on Hannchen and Excelsior

One hundred grams of seeds of each line were treated with 0.12 % formalin for 1 hour to surface sterilize the seeds and loosen the hulls. Seeds were washed vigorously for 30 min in running tap water. Water was drained off and seeds were spread thinly on paper towel to dry overnight.

Seeds were inoculated with haploid basidiospores using the spore suspension technique. All the strains (Mat-1 and Mat-2 of each isolate) were grown on PDA plates at room temperature (RT) wrapped with parafilm for 2-3 days. After that these strains were grown separately in Potato Dextrose Broth (PDB), shaken for 24 hrs at RT. An equal amount of Mat-1 and Mat-2 of each isolate was added to fresh PDB, shaken and allowed to grow for

48 hrs at RT. These cultures were used to inoculate the surface sterilized seeds using the spore suspension technique, allowed to dry for 48 hrs and then seeded in the pots.

The mixture of isolates (the inoculum, we were using) was also used to inoculate the seeds where teliospores were used as inocula. There was one control treatment (surface sterilized seeds but uninoculated).

There were 5 replications of each line with each treatment and five seeds were sown in each pot. The disease was evaluated as % infected plants. A plant showing one or more smutted heads was considered to be infected. In each pot, the number of infected plants and total plants were counted and percentage of infected plants was calculated.

Results:

This experiment was conducted in the summer of 2001 in the greenhouse and field by using teliospores as the source of inoculum. There was very poor infection and the exp. was repeated in the Fall 2001 in the greenhouse by using haploid basidiospores of different mating types of each isolate. The barley cultivars/lines showing unexpected results were tested again in Summer 2002 to confirm their reactions. The results are presented in Table 17.

There was no infection in the uninoculated seeds (control) indicating that seeds were effectively surface sterilized. CDC Candle was the most susceptible hulless line and Harrington was the most susceptible hulled line.

The mixture, we have been using is different from the genetically defined races sent by Dr Bakkeren as many cultivars showed a differential reaction with different isolates. TR640 was always resistant to the mixture but showed susceptibility to many isolates having avirulence genes V2 and V6. The results can be summarized as follow:

Cvs having *UhR1* only: SB93763, Manley, HB334, CDC Gainer, TR337, TR640, CDC McGwire, TR251, Baronesse, TR336, HB353, TR306, TR255, CDC Freedom

Cvs having *UhR1* & *UhR2*: Q21861, SM89010, TR246, TR244, CDC Helgason

Cvs having *UhR1* & *UhR6*: CDC Dolly

Cvs having *UhR1*, *UhR2* & *UhR6*: TR246, TR244?

Cvs without *UhR1*, *UhR2* & *UhR6*
HB328, RFLP Harrington, HB348, CDC Candle

Most of the present day barley cultivars carry the resistance gene; *UhR1* and some have *UhR2* or *UhR6* in addition to the *UhR1*. CDC Candle, HB328, HB348 and RFLP Harrington were susceptible to all the isolates. No barley cultivar/line was immune to all.

Table 17: Screening of barley cultivars/lines with isolates of *Ustilago hordei* in the greenhouse

	Per cent infected plants						
Genotype/Isolate	**Uh2***	**Uh3**	**Uh4**	**Uh6**	**Uh5**	**Uh 1**	**Mixture**
Q21861	0.0	0.0	4.5	14.3	0.0	5.3	13.0
SM 89010	0.0	0.0	9.1	0.0	0.0	0.0	15.0
TR244	0.0	0.0	4.5**	4.8	0.0	0.0	0.0
HB328	44.4	42.8	36.8	45.0	75.0	57.9	44.4
CDC Dolly	0.0	5.3	5.9	0.0	0.0	0.0	0.0
RFLP Harrington	23.5	15.4	18.7	10.5	6.7	18.7	52.6
TR246	0.0	0.0	5.0	0.0	0.0	0.0	10.5
SB93763	0.0	15.4	17.6	47.6	0.0	9.5	33.3
Manley	0.0	25.0	10.0	17.4	0.0	5.5	25.0
HB334	0.0	15.8	10.5	33.3	0.0	9.5	13.3
CDC Gainer	0.0	50.0	31.2	27.8	0.0	17.6	20.0
TR337	0.0	10.5	17.6	11.1	0.0	8.3	36.8
TR640	0.0	12.5	8.3	34.8	0.0	20.0	0.0
TR255	0.0	7.1	0.0	13.0	0.0	4.2	10.5
CDC McGwire	0.0	66.7	78.6	69.2	0.0	22.2	33.3
CDC Freedom	0.0	40.0	0.0	23.1	0.0	12.5	33.3
HB348	7.1	12.5	14.3	15.4	62.5	25.0	75.0
TR251	0.0	13.3	5.5	10.0	0.0	0.0	5.3
Baronesse	0.0	18.7	13.0	17.4	0.0	27.8	20.0
TR336	0.0	11.1	0.0	9.1	0.0	0.0	10.0
CDC Candle	25.0	75.0	50.0	53.8	50.0	46.1	60.0
CDC Helgason	0.0	0.0	8.7	5.3	0.0	0.0	0.0
HB353	0.0	33.3	28.6	31.2	0.0	12.5	0.0
Odessa	6.2	7.7	6.7	35.7	30.8	7.7	41.7
TR306	0.0	17.4	4.8	9.1	0.0	0.0	31.2
Hannchen	0.0	25.0	17.6	27.8	0.0	30.8	14.3
Excelsior	12.5	0.0	0.0	0.0	0.0	12.5	7.1
Plush	46.7	33.3	0.0	0.0	0.0	47.4	54.5

* Uh2 - V1V1v2v2v6v6 - avirulent on cv. Hannchen (UhR1)
Uh3 - v1v1V2V2v6v6 - avirulent on cv. Excelsior (UhR2)
Uh4 - v1v1V2V2V6V6 - avirulent on cv. Excelsior (UhR2) and cv. Plush (UhR6)
Uh6 - v1v1v2v2V6v6 - avirulent on cv. Plush (UhR6)
Uh5 - V1V1V2V2V6V6 - avirulent on Hannchen, Excelsior and Plush
Uh1 - v1v1v2v2v6v6 - virulent on Hannchen, Excelsior and Plush
Mixture - mixture of isolates from Saskatoon
**Only 1/4th part of a spike was infected

- Uh3 – Smutted curly head symptoms are common with this isolate
- Uh4 – Symptoms on flag leaves particularly on TR640 and Baronesse were common
- Uh6 – Symptoms on leaves were common and some infected heads were deformed to leaves

It confirms our early findings that the resistance in barley cultivars to covered smut is not complete. However, there are many cultivars viz. TR244, Q21861, TR246, CDC Dolly, CDC Helgason which gave very good resistance to the most of the isolates and can be used as sources of resistance. There is need to identify molecular markers linked to different sources of resistance and can be used to pyramid many covered smut resistance genes in elite breeding lines.

5.1.1 *Inheritance of resistance studies with the specific isolates*

Two doubled haploid populations were selected to study the inheritance of resistance with the specific isolates harbouring known avirulence genes.

Harrington/TR306 (HT) population: 150 doubled-haploid lines from the HT population used in the North American Barley Genome Mapping Project (NABGMP) were inoculated with the isolate Uh 2 (V1V1v2v2v6v6). This isolate is avirulent on Hannchen (*UhR1*) and virulent on Excelsior (*UhR2*) and Plush (*UhR6*). TR306 was resistant and Harrington was susceptible to this isolate (Table).

HB328/TR244: 150 anther-culture derived DH lines from this population were inoculated with Uh 6 (v1v1v2v2V6v6). This isolate is avirulent on Plush (*UhR6*) and virulent on Hannchen (*UhR1*) and Excelsior (*UhR2*). TR244 was resistant and HB328 was very susceptible to this isolate.

The preparation of inoculum and inoculation procedure was as described earlier. 15 g seeds of each line were inoculated. The experiments were done in the field in Summer 2002. There were 2 replications of each line (one row = one replication). Each row was 12 feet in length. CDC Candle, Odessa, Hannchen, Excelsior and Plush were used as checks. The parents and checks were widely distributed. In every 56 rows, there were two rows of each parent and one row of each check.

The level of covered smut infection was evaluated as the percentage of infected heads. Two 1 m sections were measured within each row. In each 1 m section, counts of the total number of heads and number of infected heads were conducted. The two counts were averaged together for each replication and the percentage of infected heads was calculated.

Results

Harrington/TR306 population: The isolate Uh2 behaved as expected; it was avirulent on Hannchen and virulent on Excelsior, Plush and Odessa. It was virulent on Harrington and avirulent on TR306. The lines showing any infected head were classified susceptible and lines showing no infected head were classified resistant as the resistant parent, TR306 was completely free from infection. Based on this classification, resistant and susceptible lines segregated into 81: 69 not significantly different from 1: 1 ratio (χ^2= 0.96, P =0.33) indicating a single major gene (*UhR1*) in TR306. It confirmed our earlier findings that TR306 has *UhR1* only.

HB328/TR244: Hannchen, Odessa and CDC Candle were infected with Uh6 whereas, Plush and Excelsior were free from disease. HB328 showed high susceptibility (6.6 %) and in TR244, we could find only one infected head among the 14 rows (4 m each). This is in agreement of our greenhouse results. We used a cut-off point of 6 % infected heads to differentiate the resistant and susceptible lines. Based on this classification, resistant and susceptible lines segregated into 128:22 not significantly different from 7:1 ratio (χ^2= 0.64 , P =0.42) indicating three genes for resistance in TR244. The DH lines possessing all three genes had resistance equal to TR244, whereas lines possessing one or two genes had levels of resistance intermediate between TR244 and HB328. The lines with no resistance gene showed infection similar to HB328.

5.2 Generation advancement in resistant x resistant and susceptible x susceptible crosses

(i) Resistant X Resistant

Objective:
To observe whether the resistant gene(s) present in resistant lines are same or different.
In summer 2000, the following crosses were made:

- Q21861 X CDC Dolly 6
- Q21861 X TR246
- Q21861 X Manley
- TR244 X CDC Dolly 6
- Q21861 X TR244

Crossed seeds of these crosses were sent to New Zealand in the Fall of 2000. F_2 plants were grown in bulks in the Preston field in 2001. Two hundred and fifty heads were harvested from each of first four populations and 150 heads were harvested from Q21861 X TR244 populations.

To confirm the segregation of F_2 populations, they were screened with the markers polymorphic between the parents using leaf disc template method.

Population	Polymorphic Marker
Q21861 X CDC Dolly 6	aHor2
Q21861 X TR246	UhR 450
Q21861 X Manley	aHor2
TR244 X CDC Dolly 6	Un8 700R
Q21861 X TR244	aHor2

By using molecular markers, segregation was confirmed in all the populations. All populations were segregating as expected except for Q21861 X TR244 that may be a partial self. The first three populations were sown in the greenhouse by taking one seed from each head (SSD) for advancement. F_5 seeds from these populations were grown in field in Summer 2002 for seed increase. These populations will be ready for marker and phenotypic study in 2003.

The following crosses were made in the spring of 2001 and F_1 seedlings were grown in the field and seeds were harvested.

- TR640 X TR244
- TR255 X Q21861

F_2 seeds of these crosses were sent to New Zealand in the Fall of 2001 and 250 heads and some bulk seed from each population were harvested. In Summer 2002, single seed hills from individual heads and bulk plots from bulk seeds were planted to advance the generations.

(ii) Susceptible X Susceptible parents

Objective:
It has been observed that some susceptible parents do not show complete susceptibility. They may have some minor genes for resistance to covered smut. If two susceptible parents are crossed, these minor genes may complement and give rise to some resistant segregants.

The following crosses were made:

- RFLP Harrington X SM89010
- RFLP Harrington X HB328

These crosses were made in the spring of 2001 and F_1 seedlings were grown in the field in summer of 2001 and seeds were harvested. F_2 seeds of these crosses were sent to New Zealand in the Fall of 2001 and 250 heads from each population were harvested. In Summer 2002, single seed hills from individual heads and bulk plots from bulk seeds were planted to advance the generations.

5.3 Transfer of true loose smut and covered smut resistance to CDC McGwire through MAS

CDC McGwire (a barley variety from Crop Development Centre, University of Saskatchewan, Saskatoon feed barley program) is a very high yielding hulless barley variety but susceptible to true loose smut and moderately susceptible to covered smut. Screening for these diseases is time, labour and space consuming and escapes are very frequent thus making it necessary to screen the resistant lines several times to confirm their resistance. As both the diseases are floral infecting, screening for them simultaneously is not possible. Molecular Marker Assisted Selection is the best alternative to combine the resistance to both the diseases in the same background.

A SCAR marker linked to the loose smut resistance gene *Un 8* has been developed and is routinely used to select loose smut resistant lines in the barley breeding program of CDC, University of Saskatchewan (Eckstein *et al.* 2002). Similarly, a SCAR marker linked to covered smut resistance in Q21861 and TR640 has been developed in this project (Ardiel *et al.* 2002).

We already have loose smut resistant lines in CDC McGwire background with resistance from TR251 (having the *Un 8* gene). *Un 8* confers resistance to most known races of *U. nuda* in western Canada and is present in the majority of resistant barley cultivars (Thomas and Menzies, 1997). Similarly, covered smut resistant lines in CDC McGwire background are available with resistance from Q21861 and TR640. A program has been initiated to combine the loose smut resistance (*Un 8*) and covered smut resistance (*Ruh q*) in CDC McGwire.

Materials and Methods:

To combine loose smut and covered smut resistance in CDC McGwire background, the following lines were selected for crossing:

Loose smut resistant lines: These lines are resistant to loose smut and the *Un 8* SCAR marker is positive.

CDC McGwire X TR251

H00752	AC9705-28	01SFBYT# 7-26
H00763	AC9705-184	01SFBYT# 8-17
H00764	AC9705-221	01SFBYT# 8-18
B00712	AC9705-84	01SFBYT# 1-36

Covered smut resistant lines: SCAR marker UhR 450 is positive in these lines and lines are resistant to covered smut.

CDC McGwire X TR640

CROSS # 8-8	MC9818-025	SH01648	01PFBYT # 38-36
CROSS # 8-82		SH01477	01PFBYT # 33-28

CDC McGwire X Q21861

CROSS # 9-1	SH01470	01PFBYT # 33-21
CROSS # 9-81	SH01474	01PFBYT # 33-25

The loose smut resistant lines were crossed with the covered smut resistant lines in all possible combinations. We followed two strategies to introgress covered smut and loose smut into CDC McGwire i.e. Doubled haploidy and backcrossing.

Doubled haploidy

F_1 seeds from the cross H00752 X 9-1 were used to produce doubled haploids (DH). Fifty one DH plants were produced using Microspore culture and these plants were tested with UhR 450 and Un 8 SCAR markers. Out of 51 plants, 20 were positive to both the markers and 12 were negative. UhR 450 was positive in 27 plants and Un 8 was positive in 32 plants. We got seeds from 40 plants and the rest were sterile. The seeds from 40 plants were planted in the greenhouse and were also send to New Zealand for seed increase. These plants will be tested against covered smut and loose smut.

Marker-assisted backcrossing

Initially we got few crossed seeds. Ten F_1 plants were grown in the greenhouse and tested with both the markers. Nine plants out of 10 were positive to both the markers (Fig. 8) and 5 plants were backcrossed with CDC McGwire. We also made some new crosses from the above mentioned list. In Summer 2002, 40 F_1 plants and 139 BC_1 F_1 plants were seeded in the greenhouse and tested with both the markers. All the F_1 plants were positive to both markers indicating that they were crossed seeds. BC_1 F_1 plants were segregated into 1:2:2:1 ratio (Table 18) indicating the presence of two major genes.

Table 18: Genotyping of BC_1 F_1 plants with Un 8 and UhR 450 markers

Generation	Total plants screened	Positive to both markers	Un 8 only	UhR 450 only	None
BC_1 F_1	139	24	66	60	37
BC_2 F_1	155	35	78	68	44

Ten F_1 and 14 BC_1 F_1 plants positive to both the markers were backcrossed with CDC McGwire. In Fall 2002, 155 BC_2 F_1 plants were screened with both the markers and 35 plants were positive for both markers (Table 18). BC_2 F_1 plants were also segregated into 1:2:2:1 ratio. The plants positive to both markers will be backcrossed to CDC McGwire. MAS will continue followed by selfing to fix the lines. After third backcross, the plants/lines positive to markers will be screened against covered smut and loose smut. The lines resistant to both the diseases will be tested for agronomic traits.

11. Publications

1. G.S.Ardiel, T.S. Grewal, P. Deberdt, B.G. Rossnagel, G.J. Scoles. 2002. Inheritance of resistance to covered smut in barley and development of a tightly linked SCAR marker. Theor Appl Genet 104:457-464.

2. Grewal, T.S., B.G. Rossnagel and G.J. Scoles. 2002. Mapping of a covered smut resistance gene to the short arm of barley (*Hordeum vulgare* L.) chromosome 5 (1H) and its linkage to adjacent markers. *Theor. Appl. Genet.* (communicated).

3. Grewal, T.S., B.G. Rossnagel and G.J. Scoles. 2002. Validation of molecular markers linked to covered smut (*Ustilago hordei*) resistance in hulless barley. Molecular Breeding (In preparation).

4. Grewal, T.S., B.G. Rossnagel and G.J. Scoles. Effect of seeding date, dehulling, inoculum age and inoculation time on the development of covered smut. Canadian J Plant Pathol. (In preparation).

Papers presented in the conferences

1. Grewal, T.S., B.G. Rossnagel and G. J. Scoles. 2002. Genetics of resistance to covered smut in barely and development of molecular markers. Annual Meeting of Genetics Society of Canada in Saskatoon, SK on June 13-15, 2002.

2. Grewal, T.S., B.G. Rossnagel and G.J. Scoles. 2002. Mapping of a covered smut resistance gene to the short arm of barley (*Hordeum vulgare* L.) chromosome 5 (1H). International Triticeae Mapping Initiative (ITMI) workshop in Winnipeg, MB on June 1-4, 2002.

3. Grewal, T.S., G.S. Ardiel, P. Deberdt, B.G. Rossnagel and G.J. Scoles. 2000. Inheritance study and SCAR marker development linked to covered smut in barley. Canadian Phytopathological Society regional annual meeting held at Saskatoon Research Centre, AAFC, Saskatoon, SK on Nov 27, 2000.

(f) Project developed materials – Copy of the poster presented at ITMI workshop in Winnipeg.

(g) Project photos – N/A

(h) Acknowledgement – SAF/ADF is acknowledged during tours of the laboratory and in presentations and publications

Appendix 1: Covered smut reaction and marker data of Q21861/SM 89010 population

Parents and Q/SM lines	SCAR Marker UhR 450 A = band present B = band absent	AFLP Marker E-AAA, M-CCC A= band present B = band absent	aHor 2 A = 1100bp band B = 800bp band	$OPO6_{780}$ A = 780bp band present B = 780bp band absent	Covered Smut Reaction A = Resistant B = Susceptible
Q21861	A	A	A	A	A
SM89010	B	B	B	B	B
Q/SM-001	A	A	A	A	A
Q/SM-002	A	A	A	A	A
Q/SM-004	A	A	A	A	A
Q/SM-005	A	A	A	A	A
Q/SM-008	B	B	B	B	B
Q/SM-010	B	B	B	B	B
Q/SM-011	A	A	A	A	A
Q/SM-012	B	B	B	B	A
Q/SM-013	A	A	A	A	A
Q/SM-014	A	A	A	A	A
Q/SM-015	A	A	A	A	A
Q/SM-016	A	A	A	A	A
Q/SM-017	A	A	A	A	A
Q/SM-018	A	A	A	A	A
Q/SM-019	A	A	A	A	A
Q/SM-020	B	B	B	B	B
Q/SM-024	A	A	A	A	A
Q/SM-026	B	B	B	B	B
Q/SM-028	A	A	A	A	A
Q/SM-029	A	A	A	A	A
Q/SM-030	A	A	A	A	A
Q/SM-031	A	A	A	A	A
Q/SM-033	B	B	B	B	B
Q/SM-035	A	A	A	A	A
Q/SM-036	A	A	A	A	A
Q/SM-037	A	B	A	A	B
Q/SM-038	A	A	A	A	A
Q/SM-039	B	A	B	B	A
Q/SM-041	B	A	B	B	B
Q/SM-042	A	B	B	B	A
Q/SM-043	B	B	B	B	B
Q/SM-045	A	A	A	A	A
Q/SM-046	A	A	A	A	A
Q/SM-047	A	A	A	A	A
Q/SM-048	B	B	B	B	B
Q/SM-049	A	A	A	A	A
Q/SM-052	A	A	A	A	A
Q/SM-053	B	B	B	B	B
Q/SM-054	B	B	B	B	B
Q/SM-055	B	B	B	B	B
Q/SM-056	A	A	A	A	A
Q/SM-057	B	B	B	B	B

Q/SM-058	A	A	A	A	A
Q/SM-059	B	B	B	B	B
Q/SM-060	B	B	B	B	B
Q/SM-061	B	B	B	B	B
Q/SM-062	B	B	B	B	B
Q/SM-063	A	A	A	A	A
Q/SM-065	B	B	B	B	B
Q/SM-066	B	B	B	B	B
Q/SM-067	B	B	B	B	B
Q/SM-068	B	B	B	B	B
Q/SM-069	B	A	B	A	B
Q/SM-070	B	B	B	B	B
Q/SM-071	A	A	A	A	A
Q/SM-073	B	B	B	B	B
Q/SM-075	B	B	B	B	B
Q/SM-076	A	B	A	A	A
Q/SM-077	A	A	A	A	A
Q/SM-078	B	B	B	B	B
Q/SM-079	B	B	B	B	B
Q/SM-080	B	B	B	B	B
Q/SM-083	B	B	B	B	B
Q/SM-084	A	A	A	A	A
Q/SM-085	A	A	A	A	A
Q/SM-086	B	B	B	B	B
Q/SM-088	B	B	B	B	B
Q/SM-089	A	A	A	A	A
Q/SM-090	B	B	B	B	B
Q/SM-091	A	A	A	A	A
Q/SM-093	A	A	A	A	A
Q/SM-094	B	B	B	B	B
Q/SM-097	A	A	A	A	A
Q/SM-098	B	B	B	B	B
Q/SM-099	A	A	A	A	A
Q/SM-100	B	B	B	B	B
Q/SM-101	A	A	A	A	A
Q/SM-104	B	B	A	A	B
Q/SM-105	A	A	A	A	A
Q/SM-107	B	B	B	B	B
Q/SM-108	A	A	A	A	A
Q/SM-109	B	B	B	B	B
Q/SM-110	A	A	A	A	A
Q/SM-111	A	A	A	A	A
Q/SM-112	B	B	B	B	B
Q/SM-113	A	A	A	A	A
Q/SM-114	B	B	B	B	B
Q/SM-115	A	A	A	A	A
Q/SM-116	A	A	A	A	A
Q/SM-117	A	A	A	A	A
Q/SM-118	A	A	A	A	A
Q/SM-119	A	A	A	A	A
Q/SM-120	A	B	B	A	A
Q/SM-121	B	A	B	B	B
Q/SM-122	A	A	A	A	A
Q/SM-123	A	A	A	A	A

Q/SM-124	B	B	B	B	B
Q/SM-125	A	A	A	A	A
Q/SM-126	A	A	A	A	A
Q/SM-127	B	B	B	B	B
Q/SM-128	A	B	B	A	A
Q/SM-129	A	A	A	A	A
Q/SM-130	B	A	A	A	B
Q/SM-132	B	B	B	B	B
Q/SM-133	B	B	B	B	B
Q/SM-134	A	A	A	A	A
Q/SM-135	B	B	B	B	B
Q/SM-136	B	B	B	B	B
Q/SM-137	A	A	A	A	A
Q/SM-138	A	A	A	A	A
Q/SM-139	A	A	A	A	A
Q/SM-140	B	A	B	B	B
Q/SM-141	A	A	A	A	A
Q/SM-142	A	A	A	A	A
Q/SM-143	A	A	A	A	A
Q/SM-144	B	B	B	B	B
Q/SM-145	B	A	B	A	B
Q/SM-146	A	A	A	A	A
Q/SM-147	B	B	B	B	B
Q/SM-148	A	A	A	A	A
Q/SM-149	A	A	A	A	A
Q/SM-150	B	B	B	B	B
Q/SM-155	B	B	B	B	B
Q/SM-156	A	A	A	A	A
Q/SM-157	A	A	A	A	A
Q/SM-158	B	B	B	B	B
Q/SM-159	B	B	B	B	B

Appendix 2. Covered smut incidence and marker data on CDC Candle/Q21861 population

Test	% infected plants			Disease	UhR 450*	aHor 2**	OPO 6***
	Spring 01	Spring 02	Summer 02	Reaction			
CDC Candle	72.72	70.00	70.00	S	No	A	No
Q21861	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-001	Ng	100.00		S	No	A	No
C/Q-002	0.00	20.00	0.00	R	Yes	B	Yes
C/Q-003	0.00	41.66		S	No	A	No
C/Q-004	75.00			S	No	A	No
C/Q-005	0.00	100.00		S	No	A	Yes
C/Q-006	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-007	0.00	62.50		S	No	A	No
C/Q-008	0.00	12.50	0.00	R	Yes	B	Yes
C/Q-009	20.00	8.33	8.33	R	No	A	No
C/Q-010	0.00	10.00	0.00	R	Yes	B	Yes
C/Q-011	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-012	16.67	7.69	0.00	R	Yes	A	No
C/Q-013	0.00	33.33		S	No	A	No
C/Q-014	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-015	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-016	0.00	75.00		S	No	A	No
C/Q-017	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-018	20.00	0.00	0.00	R	Yes	B	Yes
C/Q-019	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-020	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-021	12.50	0.00	0.00	R	No	A	No
C/Q-022	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-023	0.00	36.36		S	Yes	B	Yes
C/Q-024	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-025	14.30	0.00	0.00	R	Yes	B	Yes
C/Q-026	Ng	100.00		S	No	A	No
C/Q-027	0.00	0.00	0.00	R	No	A	No
C/Q-028	0.00	8.33	0.00	R	Yes	B	Yes
C/Q-029	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-030	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-031	0.00	66.66		S	No	A	No
C/Q-032	0.00	7.69	0.00	R	No	A	No
C/Q-033	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-034	0.00	0.00	0.00	R	Yes	A	No
C/Q-035	100.00			S	No	A	No
C/Q-036	25.00			S	No	A	No

C/Q-037	0.00	0.00	0.00	R	Yes	A	Yes
C/Q-038	0.00	20.00	0.00	R	Yes	B	Yes
C/Q-039	0.00	0.00	27.27	S	No	A	No
C/Q-040	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-041	0.00	100.00		S	No	A	No
C/Q-042	60.00			S	No	A	No
C/Q-043	0.00	6.67	25.00	S	No	A	No
C/Q-044	0.00	0.00	21.42	S	No	A	No
C/Q-045	20.00	18.18	0.00	R	Yes	B	Yes
C/Q-046	0.00	8.33	9.09	R	Yes	B	Yes
C/Q-047	0.00	0.00	0.00	R	No	A	Yes
C/Q-048	0.00	12.50	0.00	R	Yes	B	Yes
C/Q-049	50.00			S	No	A	No
C/Q-050	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-051	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-052	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-053	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-054	60.00			S	No	A	No
C/Q-055	14.30	9.09	9.09	R	Yes	B	Yes
C/Q-056	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-057	66.66			S	No	A	No
C/Q-058	40.00			S	No	A	No
C/Q-059	0.00	18.18	0.00	R	Yes	B	Yes
C/Q-060	42.10			S	No	A	No
C/Q-061	0.00	83.33		S	No	A	No
C/Q-062	0.00	20.00	58.33	S	Yes	B	Yes
C/Q-063	0.00	14.28	10.00	R	Yes	B	Yes
C/Q-064	33.00			S	No	B	Yes
C/Q-065	0.00	20.00	0.00	R	Yes	B	Yes
C/Q-066	0.00	9.09	0.00	R	Yes	B	Yes
C/Q-067	0.00	72.72		S	Yes	B	Yes
C/Q-068	37.50			S	No	A	No
C/Q-069	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-070	37.50			S	No	A	No
C/Q-071	62.50			S	No	A	No
C/Q-072	62.50			S	No	A	No
C/Q-073	25.00"	18.18	0.00	R	Yes	B	No
C/Q-074	100.00			S	No	A	No
C/Q-075	0.00	9.09	0.00	R	Yes	B	Yes
C/Q-076	0.00	0.00	0.00	R	No	A	No
C/Q-077	0.00	54.54		S	No	A	No
C/Q-078	50.00			S	No	A	No
C/Q-079	50.00			S	No	A	No
C/Q-080	0.00	7.28	0.00	R	Yes	B	Yes

C/Q-081	33.33			S	No	A	No
C/Q-082	0.00	60.00		S	No	A	No
C/Q-083	14.30	16.67	12.50	R	Yes	B	Yes
C/Q-084	33.33	75.00		S	Yes	B	Yes
C/Q-085	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-086	0.00	40.00		S	No	A	No
C/Q-087	25.00	66.67		S	No	A	No
C/Q-088	0.00	16.67	0.00	R	Yes	B	Yes
C/Q-089	100.00			S	No	A	No
C/Q-090	44.44			S	No	A	No
C/Q-091	33.33"	8.33	12.50	R	Yes	B	Yes
C/Q-092	0.00	22.22"	18.18	R	Yes	B	Yes
C/Q-093	0.00	0.00	8.33	R	Yes	B	No
C/Q-094	0.00	36.36		S	No	A	No
C/Q-095	0.00	20.00	0.00	R	Yes	B	Yes
C/Q-096	0.00	16.67	11.11	R	Yes	B	Yes
C/Q-097	0.00	70.00		S	No	A	No
C/Q-098	0.00	12.50	10.00	R	Yes	B	Yes
C/Q-099	16.67	37.50		S	Yes	B	Yes
C/Q-100	0.00	55.55		S	No	A	Yes
C/Q-101	33.33	0.00		S	No	B	No
C/Q-102	66.67	27.27		S	Yes	B	Yes
C/Q-103	0.00	16.67	18.18	R	Yes	B	Yes
C/Q-104	0.00	16.67	6.67	R	Yes	B	Yes
C/Q-105	0.00	71.42		S	No	A	No
C/Q-106	0.00	20.00	16.67	R	Yes	B	Yes
C/Q-107	0.00	33.33		S	Yes	A	No
C/Q-108	16.67	72.72		S	No	A	No
C/Q-109	0.00	10.00	0.00	R	Yes	B	Yes
C/Q-110	0.00	33.33		S	No	A	No
C/Q-111	0.00	25.00		S	Yes	A	No
C/Q-112	0.00	10.00	0.00	R	Yes	B	Yes
C/Q-113	9.09	81.81		S	No	A	No
C/Q-114	25.00	83.33		S	No	B	No
C/Q-115	0.00	16.67	0.00	R	Yes	B	Yes
C/Q-116	0.00	12.50	54.54	S	No	A	No
C/Q-117	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-118	25.00	66.67		S	No	A	No
C/Q-119	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-120	20.00	100.00		S	No	A	No
C/Q-121	0.00	20.00	0.00	R	Yes	B	Yes
C/Q-122	0.00	40.00		S	No	A	No
C/Q-123	33.33	80.00		S	No	A	No
C/Q-124	14.28	15.38	0.00	R	Yes	A	Yes

C/Q-125	75.00			S	Yes	B	Yes
C/Q-126	50.00			S	No	A	No
C/Q-127	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-128	11.11	80.00		S	Yes	B	Yes
C/Q-129	10.00	20.00	0.00	R	Yes	B	Yes
C/Q-130	Ng	75.00		S	No	A	No
C/Q-131	11.11	18.18	10.00	R	Yes	B	Yes
C/Q-132	33.33	33.33		S	No	A	No
C/Q-133	16.67	80.00		S	No	A	No
C/Q-134	60.00			S	No	A	No
C/Q-135	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-136	0.00	12.50	0.00	R	No	A	No
C/Q-137	50.00			S	No	A	No
C/Q-138	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-139	33.33			S	No	A	No
C/Q-140	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-141	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-142	10.00	0.00	0.00	R	No	A	No
C/Q-143	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-144	28.57			S	No	A	No
C/Q-145	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-146	66.67			S	No	A	Yes
C/Q-147	66.67			S	No	A	No
C/Q-148	75.00			S	No	A	No
C/Q-149	0.00	0.00	8.33	R	No	A	No
C/Q-150	16.67	41.67		S	No	A	No
C/Q-151	0.00	28.57		S	No	A	No
C/Q-152	0.00	0.00	7.14	R	Yes	B	Yes
C/Q-153	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-154	33.33	7.28		S	Yes	A	No
C/Q-155	22.22	66.67		S	No	A	No
C/Q-156	0.00	9.09	7.69	R	Yes	B	Yes
C/Q-157	0.00	7.28	21.43	S	No	A	No
C/Q-158	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-159	0.00	14.28	0.00	R	Yes	B	Yes
C/Q-160	0.00	9.09	0.00	R	Yes	B	Yes
C/Q-161	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-162	20.00	7.69	7.14	R	No	A	No
C/Q-163	0.00	0.00	0.00	R	Yes	B	Yes
C/Q-164	Ng	11.11	10.00	R	Yes	B	Yes
C/Q-165	0.00	62.50		S	No	A	No
C/Q-166	11.11	27.27		S	No	A	No
C/Q-167	0.00	50.00		S	Yes	B	Yes

Ng - not germinated
* A band of 442 bp is present
** Hor2 A- 800 bp size band is present as in CDC Candle
B- 1100 bp size band is present as in Q21861
*** OPO 6 - 780 bp band present
" Number of plants too few to determine reaction

Disease Reaction: R- lines with ≤ 20 % infected plants
S- lines with > 20 % infected plants

Theor Appl Genet (2002) 104:457–464 © Springer-Verlag 2002

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Inheritance of resistance to covered smut in barley and development of a tightly linked SCAR marker

Received: 9 January 2001 / Accepted: 31 May 2001

Abstract Inheritance of resistance to covered smut in the barley line Q21861 was studied using a doubled-haploid population produced by crossing Q21861 with the line SM89010. Based on 3 years of screening in the field and two seasons in the greenhouse, segregation for resistance/susceptibility fits a one-gene ratio, indicating a single major gene for resistance in Q21861. Of 440 random 10-mer primers tested using bulked segregant analysis, one primer (OPJ10) resulted in a reproducible polymorphic band. RAPD marker $OPJ10_{450}$ co-segregated in repulsion with the covered smut resistance. This marker was converted to a sequence-characterized amplified region (SCAR) marker linked in coupling (5.5 cM) with the covered smut resistant gene in Q21861. The SCAR marker was amplified in the line TR640 which is also resistant to covered smut, but not in the other resistant lines. The SCAR marker will be useful for marker-assisted selection for covered smut in barley breeding programs.

Keywords Barley · *Ustilago hordei* · Disease resistance · RAPD · SCAR

Introduction

Covered smut of barley (*Hordeum vulgare* L.) caused by *Ustilago hordei* (Pers.) Lagerh. occurs everywhere that barley is grown (Mathre 1997). The disease results in yield reductions ranging from 0.2 to 0.8% in Western Canada (Thomas and Menzies 1997). Economic loss is not only due to decreased yield but also to contamination of healthy seeds with black teliospores (Mathre 1997). Covered smut can be effectively controlled by fungicidal seed treatments, by sowing disease-free seed and by growing resistant cultivars. Seed treatment with fungicides is very effective but the producer must incur additional costs. In addition, the pathogen may become resistant to fungicides (Ben-yephet et al. 1975). Induced mutants of *U. hordei* tolerant to four different fungicides have been reported (Ben-yephet et al. 1975; Henry et al. 1987). Similarly, carboxin- and fenfuram-resistant strains of true loose smut (*Ustilago nuda* (Jens.) Rostr.) have been reported on winter barley crops in France (Leroux and Berthier 1988). Resistant cultivars are generally recognized as the most-economical and preferred method of control.

Understanding the inheritance of resistance to disease is valuable for planning crosses in breeding programs, identifying resistant genes and developing genetic markers to assist in selection. There are few reports on the inheritance of resistance to covered smut (Johnston 1934; Shands 1956; Wells 1958; Metcalfe 1962; Cherewick and Buchannon 1969). Johnston (1934) studied the inheritance of resistance in a cross between cultivars Trebi (moderately susceptible) and Glabron (resistant) but could not explain the genetics of resistance because of unsatisfactory infections from artificial inoculations. Other studies have reported a single dominant gene, two dominant genes or a recessive gene in different resistant cultivars/lines (Shands 1956; Wells 1958; Metcalfe 1962; Cherewick and Buchannon 1969). The different sources of resistance have different levels of expression and the level of expression may vary under different environmental conditions (Thomas 1988). Most of these early studies used segregating early generations (F_2 plants or F_3 families) to study the inheritance of resistance. However, it is often very difficult to interpret results in such genotypes because of heterozygosity and segregation, the inability to replicate such genotypes and the possibility of disease escapes. The use of homozygous lines removes heterozygosity as a source of variation and repeat testing can be carried out to confirm disease reaction (Knox et al. 1998).

Communicated by M.A. Saghai Maroof

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Mapping of a covered smut resistance gene to the short arm of barley (*Hordeum vulgare* L.) chromosome 5 (1H)

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Introduction

Mapping of an economically important trait is important as it locates the genetic locus for that trait to a particular chromosome. Other mapped loci in that region, particularly molecular markers, may be linked to the trait of interest, and could be used in molecular marker-assisted selection (MMAS) for that trait.

Covered smut of barley caused by *Ustilago hordei* (Pers.) Lagerh. occurs everywhere barley is grown. Economic loss is due to decreased yield and to contamination of grain with teliospores. This disease is a good candidate for MMAS as traditional screening is time consuming (requiring a complete generation) and thus considerable resources. Infection is inconsistent and even in highly susceptible lines some disease-free plants occur making screening difficult. Using bulked segregant analysis, we identified a RAPD marker $OPJ10_{450}$ linked to covered smut resistance in a doubled-haploid barley population from the cross Q21861 x SM89010 (Ardiel *et al.* 2002). This marker was converted to a SCAR marker (*Uhr* 450) producing a single band of 450bp in Q21861 and resistant segregates, but no band in SM89010 and susceptible segregates.

Here we report the mapping of RAPD marker $OPJ10_{450}$ to the short arm of chromosome 5 (1H) and linkage of the marker and the covered smut resistance gene to other markers present on the short arm of this chromosome (1HS).

Materials and Methods

- Q21861 X SM89010 (Q/SM): 127 DH lines.
- Harrington x TR306 (HT) 149 DH lines used in the North American Barley Genome Mapping Project (NABGMP)
- Parents of the CDC breeding populations segregating for covered smut resistance (Table 1)
- Parents of three other mapping populations viz. Steptoe/Morex (SM), Igri/Franka (IF) and Proctor/Nudinka (PN).

PCR reactions and electrophoresis were performed as reported earlier (Ardiel *et al.* 2002).

25 µl reaction volume containing 200 µM dNTP, 400 nM of each primer (KV1 and KV9), 50 ng genomic DNA, 1.5 mM $MgCl_2$, 1 unit *Taq* polymerase and 1X PCR buffer. 35 cycles of 94°C (30 s), 63°C (1 min), 72°C (1 min).

30 µg of genomic DNA cut with *EcoRI*, separated on 0.8% agarose gel and transferred to nylon membranes. The probe Act 8A (received from Dr. A. Kleinhofs, WSU, Pullman, USA) was prepared by PCR amplification and labeled with ^{32}P-dCTP using the Random Primers Labeling System. Membranes were washed to a high stringency using 1 x SSC, 0.1 % SDS at 65°C.

25 µl reaction volume containing 100 µM dNTP, 600 nM primer, 100 ng genomic DNA, 2.0 mM $MgCl_2$, 1 unit *Taq* polymerase, 1X PCR buffer. 35 cycles of 94°C (1 min), 37°C (1 min), 72°C (1.5 min).

Linkage analyses and map construction for markers and the resistance locus were performed with MAPMAKER/EXP V3.0.

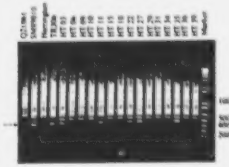

Fig. 1 DNA amplification from Q21861, SM89010, Harrington, TR306 and lines from HT population with primer OPJ10 (arrow indicates the polymorphic band). The bright polymorphic band is present in SM89010, TR306, HT1, HT11, HT22, HT27 and HT35.

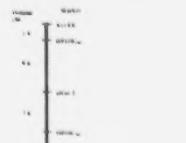

Fig. 2 A linkage map showing RAPD marker $OPJ10_{450}$ and other markers on the short arm of chromosome 5 (1H) of mapping population Harrington /TR306. Distance reported in Kosambi centiMorgans units.

Results

Primer OPJ10 (AAGCCCGAGG) was used to screen DNA of the parents of four mapping populations (HT, SM, IF, PN) as well as Q21861 and SM89010, using the PCR reaction conditions reported earlier (Ardiel *et al.* 2002). The band of interest (~450bp) was amplified from all lines, including Q21861 and SM89010. In an attempt to reproduce the polymorphism as reported by Ardiel *et al.* (2002), different primer concentrations (0.2 µM to 1.2 µM) or different $MgCl_2$ concentrations (1.0 mM to 8.0 mM) were tried. Varying primer concentration failed to change the banding pattern. However 5.0 mM $MgCl_2$ or higher consistently resulted in the original polymorphism, a bright band of ~450bp present in SM89010 with a faint band of same size present in Q21861. Therefore 6.0 mM $MgCl_2$ was used for further experiments.

The parents of the mapping populations were screened. The polymorphic band present in SM89010 was also present in TR306 and absent in Harrington. There was no polymorphism between the parents of the other mapping populations.

The HT polymorphism was reproducible (Fig. 1) and the HT mapping population (149 DH lines) was screened with primer OPJ10. This data was compared with the raw mapping data from the HT population from the GrainGenes website (http://wheat.pw.usda.gov). Visual inspection of the raw data suggested that RAPD marker $OPJ10_{450}$ was in the region of Act 8A, aflor 2 and $OPO6_{780}$ on the short arm of chromosome 5(1H). The raw data for these markers on the HT population from the GrainGenes website, together with that of $OPJ10_{450}$ was analyzed by MAPMAKER. $OPJ10_{450}$ mapped 9.6 cM from aflor 2 and 17.2 cM from $OPO6_{780}$ (Fig. 2).

The Hor 2 locus encoding B hordeins (sulphur-rich barley storage proteins) is located on 1HS. The PCR based aflor 2 polymorphism is a co-dominant STS marker producing an 800bp band in TR306 and a 1100bp band in Harrington with primers KV1 (5'-CCACCATGAAGACCTTCCTC-3') and KV9 (5'-TCGCAGGATCCTGTACAACG-3'). The PCR-based aflor 2 marker was polymorphic between parents of populations Q/SM, Q21861/CDC Candle, CDC Dolly/RFLP Harrington, and Manley/HB334 (Table 1).

Table 1 Screening of parents of covered smut segregating populations with the aflor 2 marker (PCR based co-dominant marker producing two bands at 800bp and 1100bp).

Cultivar/Line	aflor 2 (bp)	
	800bp band	1100bp band
Q21861	Yes	Yes
CDC Candle	Yes	
CDC Dolly	Yes	
RFLP Harrington	Yes	Yes
SM89010	Yes	
SM1035	Yes	
CDC Gainer		Yes
SM1036		Yes
Kannchen		Yes
SM1038		Yes
SM1042		Yes
SM1048		Yes
CDC Meredith		Yes
CDC Freedom		Yes
SM1055	Yes	
Manley	Yes	
SM1058		Yes
SM1061		Yes
SM1065	Yes	
SM1066		Yes
Harrington		Yes

Cultivars or lines not screened for covered smut

Since the aflor 2 primers produced an 1100bp band in Q21861 and an 800bp band in SM89010, the resistant and susceptible bulks and their constituents were screened with this marker to determine if it was linked to the covered smut resistance locus. The marker produced the expected 1100bp band in the resistant bulk, and in all individuals constituting the resistant bulk and produced the 800bp band in the susceptible bulk and all individuals constituting the susceptible bulk (Fig. 3). Subsequently, all lines from this population were screened with this marker. There were 11 recombinants between the marker and covered smut resistance locus.

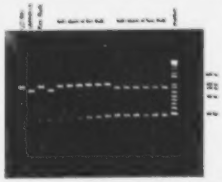

Fig. 3 Amplification products obtained with aflor 2 primers KV1 and KV9. 1100bp band is present in Q21861, the resistant bulk and individuals of the resistant bulk and 800bp band is present in SM89010, the susceptible bulk and individuals of the susceptible bulk.

Marker aflor 2 was polymorphic in two RIL populations segregating for covered smut resistance, CDC Dolly/RFLP Harrington and HB334/Manley (unpublished data). Screening resistant and susceptible lines from these populations with aflor 2 indicated that it was not linked to covered smut resistance in these populations.

The Act 8A marker (RFLP) previously mapped to the distal end of 1HS in the HT population was very close to RAPD marker $OPJ10_{450}$. However, this marker was not polymorphic between Q21861 and SM89010, therefore linkage to the covered smut resistance in Q21861 could not be determined.

Primer OPO6 (CCACGGGAAG) produced a 780bp polymorphism in the HT mapping population and was mapped to 1HS. A bright band of ~ 780bp in size was present in Q21861, the resistant bulk and all individuals of the resistant bulk and a faint band of the same size was present in SM89010, the susceptible bulk and all susceptible individuals (Fig. 4). The complete population was screened with this primer. There were 11 recombinants between this marker and the covered smut resistance locus derived from Q21861.

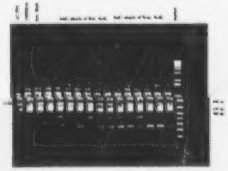

Fig. 4 Amplification products obtained with primer $OPO6_{780}$. a bright ~780bp band is present in Q21861, the resistant bulk and individuals of the resistant bulk and a faint band of the same size is present in SM89010, the susceptible bulk and individuals of the susceptible bulk.

In reviewing the covered smut reaction and marker data from the Q/SM population, the marker patterns for three markers ($OPJ10_{450}$/*Uhr* 450, aflor 2, $OPO6_{780}$) for seven lines was opposite of that expected based on the disease reaction ascribed to those lines. A review of the data for these seven lines (Table 2) failed to indicate any reason to suggest either that lines Q/SM 12 and 39 were not correctly scored as resistant or that Q/SM 37 was not correctly scored as susceptible. However, the four remaining lines (Q/SM 28, 46, 141 and 148) were classified as resistant rather than susceptible based on their reaction in the field and greenhouse.

Table 2 Covered smut reaction of 7 lines misclassified to all the markers ($OPJ10_{450}$/*Uhr* 450, aflor 2, $OPO6_{780}$).

Line #	% infected heads in field			% infected plants in greenhouse	
	1998	1999	2000	Spring 2000	Summer 2000
Q/SM 12	0.44	0.43	0.25	0.00	0.00
Q/SM 37	10.25	12.50	NP*	NT	NT
Q/SM 39	0.50	0.13	0.00	0.00	0.00
Q/SM 28	1.50	1.10	0.58	20.00	NT
Q/SM 46	0.25	2.14	NT	NT	NT
Q/SM 141	0.00	0.79	0.00	13.18	NT
Q/SM 148	0.43	2.07	NT	NT	NT

*NT- Not tested

Based on the guidelines for resistance gene nomenclature in barley (BGN 11:1-16), the covered smut resistance locus in Q21861 should be designated *Ruh q*. MAPMAKER/EXP V3.0 was used to determine the linkage of the SCAR marker *Uhr* 450, aflor 2 and $OPO6_{780}$ with the covered smut resistance locus *Ruh q* in the Q/SM population (Fig. 5).

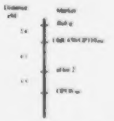

Fig. 5 A linkage map for the markers *Uhr* 450/$OPJ10_{450}$, aflor 2, $OPO6_{780}$ and *Ruh q* on the short arm of chromosome 5 (1H) in the Q/21861 X SM89010 population. Distances reported in Kosambi centiMorgan units.

Discussion

RAPD marker $OPJ10_{450}$, previously shown to be linked to covered smut resistance in Q21861 (Ardiel *et al.* 2002) was mapped to the short arm of chromosome 5(1H) using the HT mapping population. The mapping of $OPJ10_{450}$ enabled us to identify an STS marker aflor 2 and a RAPD marker $OPO6_{780}$ as also being linked to covered smut resistance.

The RAPD technique is sensitive to reaction conditions which can result in poor reproducibility. Such was the case in the present study as the polymorphism identified by Ardiel *et al.* (2002) between Q21861 and SM89010 (produced by primer OPJ10) could not initially be reproduced. However by using a higher $MgCl_2$ concentration than that used originally, the polymorphism was reproduced. This is an example of why molecular markers, that are more robust than RAPDs, are desirable for utilization in MMAS programs.

Covered smut infection is highly influenced by the environment. This can make correct classification of segregating lines difficult, particularly when a line is near to the R/S cut-off point (in this study less than 2% infected heads = resistant; greater than 2% infected heads = susceptible). In the present study seven lines had marker patterns for all linked markers that were the opposite of what would have been expected based on the disease reaction ascribed to those lines. While this might be a result of recombination between these marker loci and the disease resistance locus, it is also possible that the disease reaction ascribed to these lines was incorrect. After close inspection of the disease data from those lines, four were assumed to have been assigned the incorrect disease reaction, either because their reaction was close to the cut-off point in some years or because of incomplete disease reaction data sets. It is for exactly this reason that MMAS would be a superior method of disease screening compared to traditional techniques.

The hordein loci on 1HS have been used for cultivar identification, cladistic analysis and the study of population dynamics in barley (Kanazin *et al.* 1993). These hordein loci have also been linked to a number of pest resistance loci (e.g. powdery mildew resistance, scald resistance and Russian wheat aphid resistance) on this region of chromosome 5(1H). The leaf rust resistance gene *Rph 6* was also mapped to 1HS. This study adds the covered smut resistance locus *Ruh q* to this region. This indicates a clustering of disease resistance genes in this particular region of chromosome 1HS as suggested by Graner (1996).

Mapping disease resistance loci allows us to gain a better understanding of how the numerous resistance genes are organized within the barley genome. This information can be useful in devising breeding strategies and can provide insight into the evolution of new resistance genes and alleles. Mapping such traits, as in this study, also allows other markers to be identified. Molecular markers tightly linked to the resistance genes, like *Uhr* 450/$OPJ10_{450}$ to *Ruh q* in this study, could ultimately be used in a map-based approach to cloning the disease resistance locus, leading to a better understanding of the molecular basis of host-pathogen interaction.

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Acknowledgements

The work was funded by Saskatchewan Agriculture Development Fund Project # 19970258.

Breeding for resistance to covered smut may not be routinely performed because screening is time consuming and requires considerable resources. Plants need to be grown almost to maturity before symptom development occurs which is space and labor consuming, limiting the size of a breeding program. In addition, infection is inconsistent and even in highly susceptible lines some disease-free plants will occur (Willits and Sherwood 1999). Techniques that aid in selection for resistance, such as molecular markers, would be of value to the breeding program.

Molecular markers facilitate breeding for disease resistance as they can be used to screen material at any stage of growth and are not influenced by the environment. The use of the random amplified polymorphic DNA (RAPD) technique (Welsh and McClelland 1990; Williams et al. 1990) combined with the bulked segregant analysis (BSA) technique (Michelmore et al. 1991) has been successfully used to tag major resistance genes in barley (Barua et al. 1993; Poulsen et al. 1995; Kutcher et al. 1996; Eckstein et al. 2000b) and several other crops (Kelly 1995). For extensive and efficient use in marker-assisted selection (MAS) programs RAPD markers need to be further developed. The short random primers used in RAPD analysis usually anneal with multiple sites in different regions of the genome and thus may amplify several genetic loci. In addition, the RAPD technique is sensitive to reaction conditions, which results in poor reproducibility. To overcome the problems associated with RAPD markers and to improve their utility in MAS, longer primers have been developed from RAPD fragments (Paran and Michelmore 1993). These longer primers generate a sequence-characterized amplified region (SCAR), which can be particularly useful to follow the inheritance of the marked region of the genome. SCAR markers are preferred over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions, and they can potentially be converted into allele-specific markers. SCAR markers have been developed for many crops including barley (Deng et al. 1997; Hernandez et al. 1999; Eckstein et al. 2000a, b).

In this study, we report on the inheritance of resistance to covered smut in a doubled-haploid barley population. We describe the identification of a RAPD marker linked to covered smut resistance using bulked segregant analysis and its conversion into a SCAR marker.

Material and methods

Plant material

One hundred and twenty seven anther-culture derived doubled-haploid lines from the cross Q21861 x SM89010 were used for this study. Q21861, a barley accession of unknown pedigree from CIMMYT, Mexico, is resistant to covered smut and SM89010 (a barley line from the Crop Development Centre, University of Saskatchewan, Saskatoon, malting barley program) is moderately susceptible. CDC Candle, a hulless cultivar, was used as a susceptible check in all experiments.

The parents of the following populations from the Crop Development Centre Feed Barley breeding program were screened against *U. hordei* in the greenhouse and field: HB328/TR244, CDC Dolly/RFLP Harrington, Baronesse/TR336, SB93763/TR246, CDC McGwire/TR640, HB334/TR640, HB348/TR255, CDC Candle/Q21861, CDC McGwire/Q21861.

Inoculations, field and greenhouse experiments

For inoculation with covered smut the spore suspension technique of Tapke and Bever (1942) was used with some modifications. Thirty grams of seed of each line were inoculated with a mixture of isolates of *U. hordei* (supplied by Dr J. Menzies, Agriculture and Agri-Food Canada, Winnipeg). The barley seeds were shaken vigorously for 30 s with a spore suspension consisting of 1 g of teliospores/l of distilled water, followed by incubation at room temperature for 15 min. The suspension was decanted, the seeds were dried on paper towels at room temperature and either planted immediately or stored at 4°C for up to 2 weeks and planted at convenience.

Field experiments were conducted in the summers of 1998, 1999 and 2000 at the Preston Plots, University of Saskatchewan, Saskatoon. In 1998 and 1999, all 127 lines of the Q21861 x SM89010 cross and parents were screened against covered smut. A highly susceptible cultivar CDC Candle was used as a check. Seeding was done at the end of May and there were four replications of each line. Each replicate consisted of one row (4 m in length) of each line, and five to eight rows of each parent and check in a randomized complete block design. In the summer of 2000, only 72 putative resistant lines (those that had shown less than 2% infection in 1999) were screened in the field along with the parents and check. The same 72 lines and also seven susceptible lines, along with the parents and check were screened in the greenhouse during the spring of 2000. Sixty nine lines that exhibited less than 12% infected plants in the first greenhouse test were screened again in the greenhouse in summer of 2000. There were three replicates of each genotype. Each replicate consisted of one pot, and five seeds were planted in each pot.

Disease evaluation

In the field, the level of covered smut infection was evaluated as the percentage of infected heads. Two 1-m sections were measured within each row. In each 1-m section, counts of the total number of heads (average of 100 heads per 1-m section, with no less than 50) and number of infected heads were conducted. The two counts were averaged for each replicate and the percentage of infected heads was calculated. A cut-off of 2% covered smut-infected heads was used to classify the population (less than 2% infected heads=resistant; greater than 2% infected heads=susceptible). The cut-off was established after reviewing the disease score of the parents and the population.

In the greenhouse, the level of covered smut infection was evaluated as the percentage of infected plants. A plant showing one or more smutted heads was considered to be infected. In each pot, the numbers of infected plants and total plants were counted and the percentage of infected plants was calculated. A cut-off of 12% covered smut-infected plants was used to classify the population (less than 12% infected plants=resistant; greater than 12% infected plants=susceptible). This cut-off was established after reviewing the disease score of the parents and the population.

Bulked segregant analysis with RAPD

DNA was extracted using the CTAB method of Procunier et al. (1990) with minor modifications. The extraction was scaled down 15-fold and the tissue was ground directly in CTAB extraction buffer in a 1.5-ml Eppendorf tube without liquid nitrogen. Based on disease evaluation, covered smut resistant- and susceptible-

bulks were created each consisting of equal amounts of DNA from six individuals from resistant (Q/SM- 29, 36, 38, 47, 84 and 99) and susceptible (Q/SM- 48, 67, 68, 79, 136 and 158) lines. Four hundred and forty 10-mer random primers obtained either from the Biotechnology Laboratory, University of British Columbia, Canada, or Operon Technologies Inc., California, USA, were used for RAPD analysis of the two DNA bulks. Each reaction was performed in a total volume of 25 µl, containing 100 µM each of dATP, dTTP, dCTP and dGTP (Gibco BRL, Bethesda, USA), 0.6 µM of primer, 50 ng of genomic DNA, 2 mM of $MgCl_2$, 1 unit of *Taq* DNA polymerase (Gibco BRL) and 1×PCR buffer (Gibco BRL). Amplification was performed with a GeneAmp thermocycler (Perkin Elmer, USA) and consisted of an initial denaturation step at 94°C for 3 min, followed by 40 cycles as follows: a denaturation step at 94°C for 1 min, an annealing step at 32°C for 1 min and an extension step at 72°C for 1.5 min; ending with an extension period at 72°C for 10 min. All amplification products were separated on a 1% agarose, 0.5×TBE gel, stained with ethidium bromide and viewed under ultra-violet light.

Any primers which amplified a polymorphic DNA fragment between the bulks were then used to amplify DNA from the parents and the individual genotypes constituting the bulks. The one primer (OPJ10) that resulted in a reproducible polymorphism was used to screen the entire Q/SM population.

Cloning and sequencing of RAPD products

The polymorphic amplified product of OPJ10 produced in the susceptible genotype was excised from the agarose gel and a TA Cloning Kit (Invitrogen corporation, USA) was used for cloning, as described by the manufacturer. The recombinant plasmids were transformed into *Escherichia coli* and plated on selective media containing ampicillin and X-gal. White colonies were picked from the plate and cultured overnight. The Wizard Miniprep Kit (Promega, USA) was used for plasmid DNA extraction. To confirm the presence of the insert, PCR and restriction enzyme digestion were performed.

Sequencing of the DNA insert was performed at the Plant Biotechnology Institute, Saskatoon, SK, Canada. The sequences were aligned with the aid of the software program DNASTAR (Lasergene, USA).

SCAR design and analysis

Two primers were developed from the OPJ10 amplified sequence but there was no polymorphism between resistant and susceptible genotypes. A major band was produced in all susceptible and resistant genotypes. The bands produced in the resistant and in the susceptible genotypes were cloned and sequenced. The sequences from resistant and susceptible genotypes were aligned and compared with the aid of the computer program DNAMAN (Lynnon-Biosoft, USA), and two SCAR primers were synthesized to amplify a major band only from the resistant genotype. The sequences of the two SCAR primers were:

ULR1-F GATAAGGATGTTCCGCC, and
ULR-R CCCGAGGTCCAAAATCAG.

Amplification was performed in a total volume of 25 µl, containing 100 µM of each of dATP, dTTP, dCTP and dGTP (Gibco BRL, Bethesda, USA), 200 nM of each primer (ULR1-F and ULR-R), 50 ng of genomic DNA, 2 mM of $MgCl_2$ (Gibco BRL), 1 unit of *Taq* polymerase (Gibco BRL) and 1× Gibco BRL buffer (50 mM KCL, 20 mM Tris-HCL, pH 8.4). Amplification was performed with an Amplitron II thermocycler (Barnstead/Thermolyne, USA) and consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of: denaturation at 94°C for 1 min., annealing at 64°C for 1 min and an extension at 72°C for 1.5 min; ending with an extension at 72°C for 10 min. Amplified products were separated on a 1.25% agarose, 0.5×TBE gel, stained with ethidium bromide and viewed under ultra-violet light.

The SCAR primers were used to screen the entire Q/SM population and compared to the results obtained with OPJ10. These primers were also used to screen the parents of other crosses segregating for covered smut resistance (see earlier). With a view to mapping the marker and resistance, parents of four barley map-

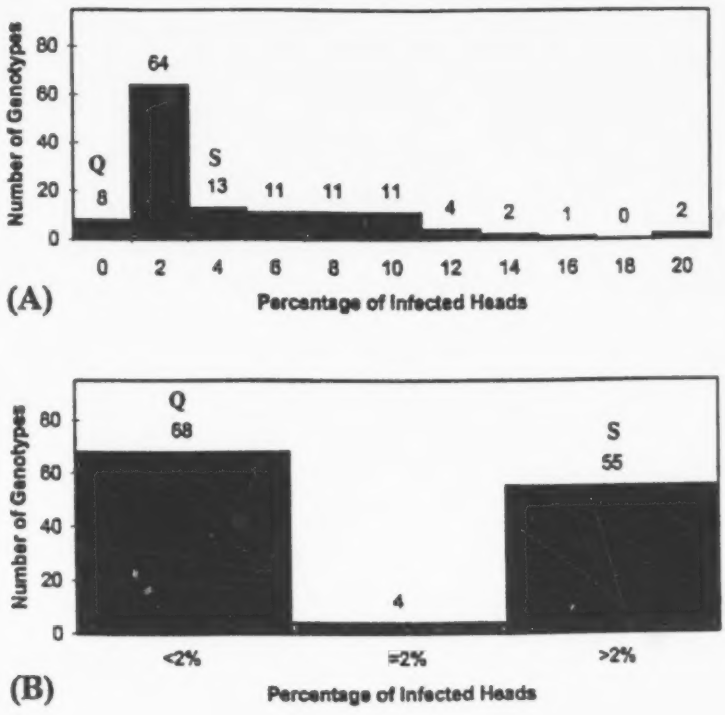

Fig. 1A, B Frequency distribution of the percentage of covered smut infected heads for the 127 lines of the doubled-haploid Q21861×SM89010 population (Q=Q21861; S=SM89010) evaluated at Preston Plots, University of Saskatchewan, August 1998, 1999. **(A)** Lines grouped at intervals of 2 for the percentage of infected heads. **(B)** Lines grouped at less than 2% infected heads, 2% infected heads and greater than 2% infected heads

Table 1 Covered smut incidence of parents, the check and the 72 putative resistant lines in field and greenhouse tests, and the presence/absence of the SCAR marker

Line	% Infected heads in field			% Infected plants in greenhouse		SCAR marker[a]	Line	% Infected heads in field			% Infected plants in greenhouse		SCAR marker[a]
	1998	1999	2000	Spring 2000	Summer 2000			1998	1999	2000	Spring 2000	Summer 2000	
CDC Candle	NT[b]	46.15	33.02	62.50	54.54	No	Q/SM-78	2.30	1.25	0.00	0.00	11.11	No
Q21861	0.40	0.25	0.10	0.00	7.14	Yes	Q/SM-84	0.00	1.21	0.17	0.00	0.00	Yes
SM89010	4.78	2.17	1.49	36.36	20.00	No	Q/SM-85	0.00	0.00	0.00	0.00	0.00	Yes
Q/SM-1	0.00	0.13	0.14	0.00	0.00	Yes	Q/SM-89	0.00	0.23	0.00	0.00	0.00	Yes
Q/SM-2	0.26	0.65	0.10	0.00	0.00	Yes	Q/SM-91	0.00	0.24	0.12	0.00	0.00	Yes
Q/SM-4	0.00	0.48	0.00	0.00	0.00	Yes	Q/SM-93	1.38	0.07	0.11	9.09	0.00	Yes
Q/SM-5	0.00	0.00	0.00	0.00	0.00	Yes	Q/SM-97	0.30	0.00	0.77	0.00	0.00	Yes
Q/SM-10	0.79	0.52	0.33	28.57	NT	No	Q/SM-99	0.00	0.19	0.00	0.00	0.00	Yes
Q/SM-11	0.48	0.08	0.00	0.00	0.00	Yes	Q/SM-101	1.14	0.00	0.00	0.00	0.00	Yes
Q/SM-12	0.44	0.43	0.25	0.00	0.00	No	Q/SM-105	0.00	0.00	0.00	0.00	0.00	Yes
Q/SM-13	0.12	1.61	1.09	10.00	0.00	Yes	Q/SM-108	0.57	0.00	0.11	0.00	0.00	Yes
Q/SM-14	0.54	0.65	0.46	0.00	0.00	Yes	Q/SM-110	0.18	0.00	1.84	0.00	0.00	Yes
Q/SM-15	0.10	1.81	0.00	9.09	0.00	Yes	Q/SM-111	0.27	0.56	0.12	0.00	0.00	Yes
Q/SM-16	0.30	0.33	0.08	0.00	0.00	Yes	Q/SM-113	1.28	0.11	1.95	0.00	0.00	Yes
Q/SM-17	0.00	0.00	0.09	0.00	0.00	Yes	Q/SM-115	1.63	1.55	0.00	0.00	0.00	Yes
Q/SM-18	0.49	0.00	0.23	0.00	0.00	Yes	Q/SM-116	0.00	0.44	0.40	0.00	0.00	Yes
Q/SM-19	0.29	0.33	0.00	0.00	0.00	Yes	Q/SM-117	0.15	0.00	0.07	0.00	0.00	Yes
Q/SM-24	1.34	0.52	1.04	9.09	0.00	Yes	Q/SM-118	0.00	0.00	1.12	9.09	0.00	Yes
Q/SM-28	1.59	1.10	0.58	20.00	NT	Yes	Q/SM-119	0.00	0.14	0.23	0.00	0.00	Yes
Q/SM-29	0.00	0.60	0.36	0.00	0.00	Yes	Q/SM-120	0.00	0.00	0.28	0.00	0.00	Yes
Q/SM-30	2.97	0.89	0.39	0.00	0.00	Yes	Q/SM-122	0.83	0.00	0.21	0.00	0.00	Yes
Q/SM-31	0.00	0.08	0.00	0.00	0.00	Yes	Q/SM-123	0.20	0.00	0.00	0.00	0.00	Yes
Q/SM-35	0.00	0.00	0.00	0.00	0.00	Yes	Q/SM-125	0.81	0.19	0.64	0.00	0.00	Yes
Q/SM-36	0.00	0.47	0.00	0.00	0.00	Yes	Q/SM-126	0.12	0.00	0.24	0.00	0.00	Yes
Q/SM-38	0.00	0.00	0.00	0.00	0.00	Yes	Q/SM-128	0.00	0.00	0.17	0.00	0.00	Yes
Q/SM-39	0.50	0.13	0.00	0.00	0.00	No	Q/SM-129	0.86	0.16	0.00	11.11	0.00	Yes
Q/SM-42	0.00	0.07	0.00	0.00	0.00	Yes	Q/SM-134	8.02	0.00	0.37	0.00	0.00	Yes
Q/SM-45	0.14	0.00	0.10	0.00	0.00	Yes	Q/SM-137	0.00	0.32	0.00	0.00	0.00	Yes
Q/SM-47	0.00	0.42	0.00	0.00	0.00	Yes	Q/SM-138	0.29	0.00	0.18	0.00	0.00	Yes
Q/SM-49	0.94	0.15	0.10	0.00	0.00	Yes	Q/SM-139	0.68	0.63	0.47	0.00	0.00	Yes
Q/SM-52	0.49	0.00	0.15	0.00	0.00	Yes	Q/SM-141	0.10	0.79	0.00	18.18	NT	Yes
Q/SM-56	0.70	1.05	0.13	0.00	0.00	Yes	Q/SM-142	1.63	0.00	0.10	0.00	0.00	Yes
Q/SM-57	1.56	1.19	1.81	16.67	NT	No	Q/SM-143	0.24	0.00	0.11	0.00	0.00	Yes
Q/SM-58	0.78	0.10	0.25	8.33	0.00	Yes	Q/SM-146	0.00	0.46	0.14	0.00	0.00	Yes
Q/SM-63	1.21	0.22	0.71	0.00	0.00	Yes	Q/SM-149	0.13	0.42	1.20	10.00	0.00	Yes
Q/SM-71	0.25	0.00	0.00	0.00	0.00	Yes	Q/SM-156	0.37	0.00	0.00	0.00	6.67	Yes
Q/SM-76	0.52	0.47	0.00	0.00	0.00	Yes	Q/SM-157	0.15	0.00	0.00	0.00	0.00	Yes
Q/SM-77	0.14	0.18	0.24	0.00	0.00	Yes							

[a] Yes=amplified band of 442 bp was present; No=band not present [b] NT=not tested

ping populations (Steptoe/Morex, TR306/Harrington, Igri/Franka, Proctor/Nudinka) were also screened with the primers.

Analysis of data

A chi-square test was used to check the fit of segregation classes. The linkage distance between the markers and the covered smut resistance gene was calculated, using percent recombination, and expressed as a recombination percentage.

Results

Inheritance study

Based on the 1998 and 1999 field data, 72 of the 127 lines in the Q/SM population were putatively classified as resistant (with less than 2% covered smut infected heads) (Fig. 1). Lines that could be clearly designated as susceptible after the first 2 years of testing were not re-tested. To confirm the resistance of the 72 lines they were tested in the greenhouse in the spring of 2000 and again in the field in the summer of 2000. Those lines that exhibited less than 12% infected plants in the greenhouse screening were re-tested in the greenhouse during the summer of 2000. The results of five separate tests on these 72 lines are summarized in Table 1. On the basis of field and greenhouse tests, 5 of the 72 lines (Q/SM-10, 28, 57, 78 and 141) previously classified as resistant were declared susceptible. Based on this classification, the resistant and susceptible lines segregated in the ratio 67:60, not differing significantly from a 1:1 ratio (χ^2=0.38; P=0.50–0.75), indicating segregation for a single major gene.

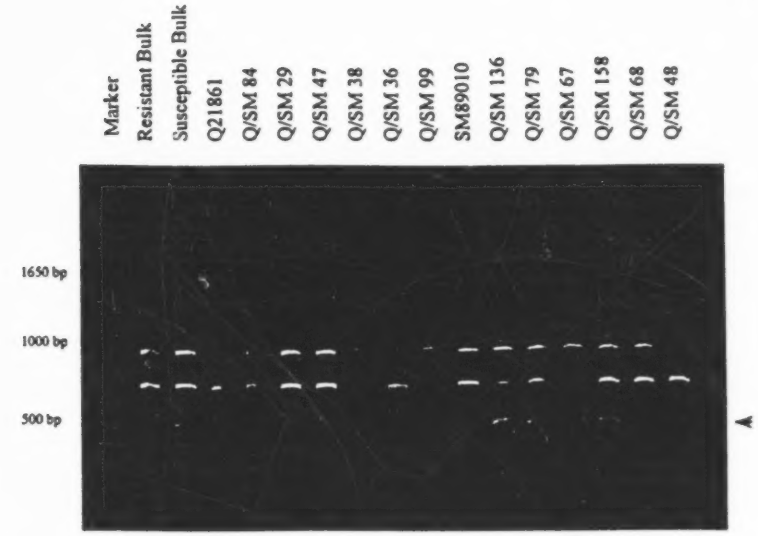

Fig. 2 DNA amplification from the covered smut-resistant bulk, susceptible bulk, resistant parent (Q21861), individuals of the resistant bulk, susceptible parent (SM89010) and individuals of the susceptible bulk with the RAPD OPJ 10 primer (*arrow* indicates polymorphic band)

The reaction of the parents of different populations to *U. hordei* (Table 2) indicated that most of the populations would be segregating for covered smut resistance. TR244, CDC Dolly, TR246, TR640 and TR255 exhibited good levels of resistance to covered smut in the field and greenhouse.

Table 2 Covered smut incidence of parents of different breeding populations in field and greenhouse tests and presence/absence of SCAR marker

Cultivar/Line	% Infected heads in field	% Infected plants in greenhouse	Scar marker[a]
TR244	1.21	11.01	No
HB328	14.60	82.37	No
CDC Dolly	0.86	5.49	No
RFLP Harrington	13.10	67.78	No
Baroness	7.31	65.38	No
TR336	1.20	26.08	No
SB93763	3.24	60.76	No
TR246	1.72	7.40	No
CDC McGwire	2.52	28.57	No
TR640	0.00	0.00	Yes
HB334	0.69	45.45	No
HB348	3.50	5.00	No
TR255	0.00	NT[b]	No
Q21861	0.25	3.57	Yes
CDC Candle	36.60	80.97	No

[a] Yes=amplified band of 442 bp was present; No=band not present
[b] NT=not tested

RAPD analysis

Of the 440 10-mer random primers tested, 97 (22%) produced no amplification product or unscoreable products seen as a smear or weak banding. A number of primers resulted in polymorphism between the bulks; however, when utilized to screen the parents and individuals constituting the bulks the polymorphic bands were either non-reproducible or not present in the parents or in a majority of the individuals in the bulks. However, the OPJ10 primer (AAGCCCGAGG) resulted in a reproducible bright polymorphic band of about 450 bp present in the susceptible bulk, the susceptible parent and each individual of the susceptible bulk (Fig. 2). The entire population was screened with the OPJ10 primer. Seven of 127 individuals (Table 3) were recombinants between the marker and the resistance gene, suggesting a linkage distance of 5.5 cM.

Development of the SCAR marker

The polymorphic band amplified by the primer OPJ10 was excised, cloned and sequenced. Two primers were developed from the amplified sequence but the same-sized band was amplified in both the susceptible and resistant parent. The band present in the resistant and susceptible genotypes was cloned and sequenced. Several primers complementary to the ends of the fragments

Table 3 The relationship between the OPJ10 RAPD polymorphism and covered smut rating

		Disease rating		
		Resistant	Susceptible	Total
DNA polymorphism Pattern	Resistant	65	5	70
	Susceptible	2	55	57
	Total	67	60	127

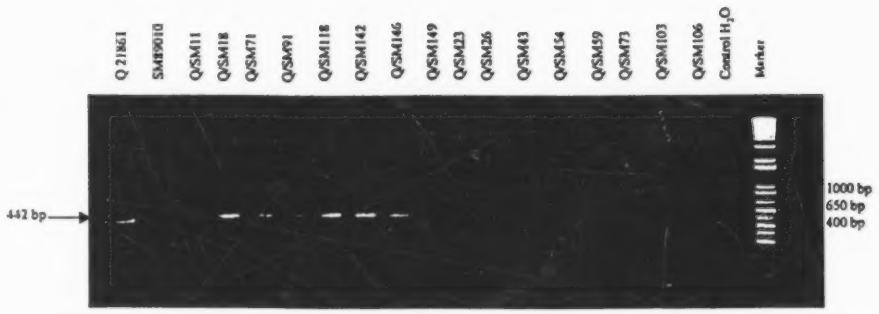

Fig. 3 Amplification product (442 bp) obtained with SCAR primers ULR1-F and ULR-R; present in resistant parent (Q21861) and resistant lines, and absent in susceptible parent (SM89010) and susceptible lines

were designed based on nucleotide differences between the two sequences. These were synthesized and tested in various combinations until a single band was produced in the resistant genotype only. The primer ULR1-F (GATAAGGATGTTCCGCC) was designed based around three nucleotide differences between the sequences of fragments from the resistant and susceptible genotypes, including the two 3′ Cs. This primer, in combination with primer ULR-R (CCCGAGGTCCAAAATCAG) which was based on an area of sequence homology between fragments of resistant and susceptible genotypes, yielded a major band of expected size (442 bp) in the resistant genotypes but not in the susceptible genotypes (Fig. 3). The polymorphic banding pattern was reproducible and the entire population was screened with these SCAR primers. The SCAR marker was found to co-segregate fully in repulsion with the OPJ10 RAPD marker.

The parents of other populations segregating for covered smut resistance and of many mapping populations of barley were screened with these SCAR primers. The 442-bp band was amplified in only Q21861 and the line TR640 and was absent in all other parents. TR640 was observed to be resistant to *U. hordei* in the field and greenhouse (Table 2).

Discussion

Screening for covered smut resistance in a breeding program is difficult, as infection by artificial inoculation is inconsistent (Willits and Sherwood 1999). The occurrence of escapes, variable disease incidence and absence of complete resistance in the resistant parent may cause difficulty in classifying segregating populations with respect to disease reaction, particularly in early segregating generations. The doubled-haploid population used in this study helped to overcome these problems in that the homozygous lines could be repeatedly tested without concern for variation arising through segregation.

In the greenhouse, infection ratings were based on the percentage of infected plants, while ratings in the field were based on the percentage of infected heads in a length of row, as individual plants could not be distinguished. A Pearson correlation was used to analyze the relationship between the percentage of infected heads and the percentage of infected plants. The percentage of infected heads and the percentage of infected plants were significantly correlated (r=0.73, P=0.000). Resource considerations did not allow single plant spacing to be used in the field. Woodward and Tingey (1941) found that when disease ratings of covered smut of barley were based on plant counts as opposed to head counts, a much higher percentage rating was obtained. This was usually the case in the present experiments (Tables 1 and 2) because often the first heads to emerge were free of infection even in susceptible plants. As every head was not infected, plant counts resulted in a higher percentage infection compared to head counts. Single-plant evaluation would be preferable to row evaluation if adequate plant numbers could be grown. However, in resistant lines, although no infected plants were found among the few plants that could be grown in the greenhouse, a few infected plants were observed among the much larger population grown in the field. As rating the disease infection as the percentage of infected plants (as opposed to the percentage of infected heads) resulted in higher levels of infection, this also resulted in a clearer delineation between resistant and susceptible lines.

Resistance in the resistant parent (Q21861) was not complete. Very low levels of infection were observed in both field and greenhouse studies (Table 1). Person and Cherewick (1964) also reported that resistance to *U. hordei* in barley was incomplete. The susceptible parent of the cross (SM89010) showed much less infection (1.49–4.78% in the field and 20–36% in the greenhouse) than the susceptible check CDC Candle (33.02–46.15% in the field and 55–63% in the greenhouse) possibly indicating some minor gene(s) for covered smut resistance in SM89010. However, the susceptible check was a hulless cultivar and hulless cultivars have been found to be more susceptible to covered smut (Ardiel 2000). CDC Candle was the most-susceptible cultivar to the source of inoculum used in this study. The high level of infection served to indicate the effectiveness of the inoculation technique.

A cut-off point of 2% was used to distinguish the resistant and susceptible classes based on the disease reaction of the susceptible parent in the 1998 and 1999 field experiments. After further testing of these putative resistant lines in the field and the greenhouse, ten lines were

moved to the susceptible category as they had exhibited a high score in the field and/or in the greenhouse tests.

The good fit of segregation classes to a 1:1 ratio would suggest that Q21861 carries a major gene for resistance to covered smut. A single dominant gene governing resistance to covered smut has been reported in many barley cultivars (Shands 1956; Wells 1958; Metcalfe 1962; Cherewick and Buchannon 1969). Some lines, viz. Q/SM- 5, 35, 38, 85 and 105, showed complete resistance (more resistant than the resistant parent) in all the tests. This may be due to the complementary effect of minor genes in SM89010 with the major gene of Q21861. The line Q/SM-134 had a very high disease score (8.02%) in only one test (field 1998), otherwise it was highly resistant in all other tests. This may be due to the carry over of seeds during seeding in the 1998 field test.

Of the 440 random primers used in the bulked segregant analysis, only one primer amplified a reproducible polymorphic banding pattern with close linkage to the covered smut resistance gene. Similarly, 540 primers were screened to identify one marker linked to the *Rpg1* gene in barley (Horvarth et al. 1995). However, Poulsen et al. (1995) screened only eight primers and identified a RAPD marker $OPU02_{2700}$ linked with a leaf rust resistance gene (*RphQ*) in Q21861 using bulked segregant analysis. On the other hand, some workers have been unable to find any reproducible polymorphisms with random primers using bulked segregant analysis in barley (Xu and Kasha 1991; Krasichynska 1996).

To overcome problems associated with using the RAPD technique for marker-assisted selection, the RAPD marker was converted into a SCAR marker. Primers were developed based on the sequence of the fragment amplified by the RAPD technique in the susceptible genotype but not the resistant genotype. These amplified a fragment of the expected length in both resistant and susceptible lines. Loss of polymorphism when primers were extended based on the sequence of a cloned RAPD fragment has been reported in other plants (Paran and Michelmore 1993; Deng et al. 1997; Hernandez et al. 1999). RAPD polymorphisms may result from mismatch at primer binding sites due to sequence divergence but also from structural changes between primer sites (Paran and Michelmore 1993). It is likely that the OPJ10 RAPD polymorphism resulted from mismatch at the priming sites, rather than structural changes. Extending primers beyond the RAPD binding site into regions of homology between the two genotypes would result in the loss of the polymorphism. Comparison of the sequences of amplified products from resistant and susceptible genotypes allowed allele-specific primers to be designed and the polymorphism between the susceptible and resistant genotypes to be recovered.

The SCAR marker was found to co-segregate with the RAPD marker. The marker was 5.5 cM from the covered smut resistant gene in Q21861. This level of linkage is generally considered to be adequate for the marker to be utilized in a breeding program for MAS (Burr et al. 1983; Mohan et al. 1997). Similarly, Eckstein et al. (2000b) identified a SCAR marker in the Q21861× SM89010 population linked with the scald resistance gene in SM89010. SCAR markers are now effectively used for MAS in routine breeding programs (Gu et al. 1995; Eckstein et al. 2000a). Mapping of the marker was not possible as the SCAR primers were unable to amplify any band in the parents of mapping populations. Attempts to develop a polymorphism (by adjusting the annealing temperature or making primers for the negative allele) for the SCAR marker in mapping populations will continue.

This is the first report of the development of a molecular marker linked with covered smut resistance in barley. The SCAR marker is closely linked to the gene of interest and can be effectively used in marker-assisted selection. This SCAR marker is also present in TR640 which is resistant to covered smut. It will be used to screen other populations involving TR640 and Q21861 as parents.

Acknowledgements We thank Dr J. Menzies, Agriculture and Agri-Food Canada, Winnipeg, for the supply of *U. hordei* teliospores. We are grateful to Peter Eckstein for his technical advice and to Doug Voth and Tom Zatorski for their assistance in field experiments. The work was funded by the Saskatchewan Agriculture Development Fund Project # 97000285.

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